Fungicide resistance and control of citrus green mould

by
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Thesis presented in fulfilment of the requirements for the degree of Master of Science in Agriculture in the Faculty of Agrisciences at Stellenbosch University

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April 2014

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Date: April 2014

SUMMARY

Green mould (caused by *Penicillium digitatum*) is a major cause of postharvest losses in citrus. The most widely used fungicides in South Africa against this disease are thiabendazole (TBZ) and imazalil (IMZ). The aim of this study was to study the optimal application of TBZ in packhouses, and to determine IMZ resistance groups in South African *Penicillium digitatum* populations in order to develop a resistance assay to quantify imazalil resistance.

Residue loading of TBZ with application methods typically used in South African packhouses for green mould control was studied. TBZ was applied curatively and protectively in aqueous dip, drench and wax coating treatments and fruit were inoculated with a TBZ-sensitive or a TBZ-resistant isolate of *P. digitatum*. The dip treatments consisted of TBZ concentrations of 0 – 2000 $\mu g.mL^{-1}$; fruit were dipped for 60 s at 22°C at a pH of 7. Residues differed between fruit batches and were 0.5 – 1.7 $\mu g.g^{-1}$ at 1000 $\mu g.mL^{-1}$ TBZ. Curative dip treatments almost completely controlled green mould (>96% at 1000 $\mu g.mL^{-1}$ TBZ). The predicted residue level needed for 75% control were 0.06 - 0.22 $\mu g.g^{-1}$ depending on citrus type. Protective treatments were unreliable and varied from 17.0 to 97.9% at 1000 $\mu g.mL^{-1}$ TBZ between fruit batches. Drench treatments consisted of exposure times of 30, 60 and 90 s with 1000 or 2000 $\mu g.mL^{-1}$ TBZ. Average TBZ-residues were 2.14 $\mu g.g^{-1}$ for Clementine mandarin fruit and 3.50 $\mu g.g^{-1}$ for navel orange fruit. Drenching of navel orange fruit resulted in 77 – 92%, 68 – 90% and 32 – 38% control for curative treatment after 6 and 24 h and protective treatments, respectively, while these treatments resulted in 66 – 84%, 34 – 56% and 9 – 17% control on Clementine mandarin fruit, respectively. Wax with 4000 $\mu g.mL^{-1}$ TBZ was applied at 0.6, 1.2 and 1.8 L $wax.ton^{-1}$ fruit. Chilling injury was evaluated after fruit storage at -0.5°C for 40 days. Average TBZ-residues loaded were 1.3, 1.3 and 2.7 $\mu g.g^{-1}$ at the recommended 1.2 $L.ton^{-1}$ for Satsuma mandarin, Clementine mandarin and Valencia orange fruit, respectively. Protective treatments showed lower infection levels (14 – 20%) than curative treatments (27 – 40%) for Valencia orange fruit. The same trend was observed with Satsuma (92 – 95% curative; 87 – 90% protective) and Clementine mandarin fruit (82 – 90% curative; 59 – 88% protective), but control was relatively poor. TBZ application in wax exceeded the MRL of 5 $\mu g.g^{-1}$ at higher wax loads (1.2 and 1.8 L/ton). Wax treatments showed a significant reduction in chilling injury; TBZ had an additive effect. Dip treatments gave better overall control of green mould, but wax application showed a better

protective treatment as well as a lower sporulation incidence. Drench treatment was much less effective than dip treatments. TBZ-resistant isolates could not be controlled.

Imazalil resistance in *P. digitatum* is a quantitative trait with multiple genes involved. Three of the better known IMZ resistance groups of *P. digitatum* have previously been characterised and are termed R1, R2 and R3. However, the relative prevalence of these groups in IMZ-resistant *P. digitatum* populations was largely unknown. In this study, 230 IMZ resistant *P. digitatum* isolates, which were obtained from green moulded citrus fruit from South Africa, the USA, Uruguay, Spain, Israel, Cyprus, Chile, Australia and Argentina, were classified into IMZ resistance groups using a multiplex PCR assay employing previously published primers to distinguish between these groups. A total of 189 isolates yielded PCR products that could be separated and visualised using gel electrophoresis. Forty-one isolates shown to be resistant could not be classified during this assay, which indicates that other uncharacterised resistant genotypes might be involved in IMZ resistance in *P. digitatum*. Isolates from the USA showed the most diversity with 13.7% of 73 isolates identified as R1, 12.3% as R2 and 56.2% as R3 type resistance, and 17.8% as unclassified. From Chile, 1.5% of 67 isolates were R1, 76.1% were R3 and 22.4% were of an unknown genotype. The 90 isolates from the other countries were all classified as R3-type resistance (83.3%) or unclassified (16.7%). Given its predominance, the R3-gene could therefore be used as target gene to quantify IMZ resistance in *P. digitatum* populations using real-time PCR. Exposed plate assays to quantify IMZ resistant spores in packhouses proved to be unreliable and a real-time PCR methodology using *P. digitatum* and R3-group specific primers were developed. The qPCR assay was optimized for mycelial DNA but was not sensitive enough for spores from environmental samples.

OPSOMMING

Groenskimmel (veroorsaak deur *Penicillium digitatum*) is een van die belangrikste oorsake van na-oes verliese by sitrus. Die fungisiedes wat meestal in Suid-Afrika teen hierdie siekte gebruik word, is thiabendasool (TBZ) en imazalil (IMZ). Die doel van die studie was om die optimale aanwending van TBZ in pakhuise te bestudeer, asook om Suid-Afrikaanse *Penicillium digitatum* populasies te klassifiseer volgens IMZ-bestandheidsgroepe, met die oog op ontwikkeling van 'n toetsmetode om IMZ-bestandheid te kwantifiseer.

Residu-lading van TBZ met aanwendingsmetodes wat tipies in Suid-Afrikaanse pakhuise vir groenskimmelbeheer gebruik word, is bestudeer. TBZ was genesend sowel as voorkomend aangewend as doop-, stort- en wakslaagbehandelings; vrugte was met 'n TBZ-sensitiewe of TBZ-bestande isolaat van *P. digitatum* geïnokuleer. Die doopbehandelings was 60-s dompelings in verskeie TBZ konsentrasies vanaf 0 tot 2000 $\mu g.mL^{-1}$ teen 22°C met 'n pH van 7. Residue het tussen vrugtipes verskil en het waardes van 0.5 – 1.7 $\mu g.g^{-1}$ gehad. Die genesende behandelings het groenskimmel grootliks beheer (>96% teen 1000 $\mu g.mL^{-1}$ TBZ). Die voorspelde residu-lading wat benodig word vir 75% beheer was 0.06 – 0.22 $\mu g.g^{-1}$ afhangend van vrugtipe. Voorkomende behandelings het tot onbetroubare beheer gelei, en het gewissel van 17 tot 97.9% teen 1000 $\mu g.mL^{-1}$ TBZ tussen stelle vrugte. Stortbehandelings het bestaan uit blootstellingstye van 30, 60 of 90 s met 1000 of 2000 $\mu g.mL^{-1}$ TBZ. Die gemiddelde residu waardes was 2.14 $\mu g.g^{-1}$ op Clementine naartjies, en 3.50 $\mu g.g^{-1}$ op nawellemoene. Op nawels het stortbehandelings onderskeidelik 77 – 92% en 68 – 90% genesende beheer na 6 en 24 ure gegee en 32 – 38% voorkomende beheer; op Clementine naartjies was beheer onderskeidelik 66 – 84%, 34 – 56% en 9 – 17%. Waks is teen 0.6, 1.2 en 1.8 L waks per ton vrugte teen 4000 $\mu g.mL^{-1}$ TBZ toegedien. Wakslaagbehandelde vrugte is ook vir koueskade ná opberging teen -0.5°C vir 40 dae geëvalueer. Die gemiddelde residu-waardes op Satsuma naartjies, Clementine naartjies en Valencia lemoene was 1.3, 1.3 en 2.7 $\mu g.g^{-1}$ teen die aanbevole 1.2 L waks per ton, onderskeidelik. Voorkomende behandelings het laer infeksievlakke (14.1 – 19.8%) as genesende behandelings (27 – 40%) op Valencia lemoene gehad. Dieselfde het vir Satsuma (92 – 95% genesend; 87 – 90% voorkomend) en Clementine nartjies (82 – 90% genesend; 59 – 88% voorkomend) gegeld, maar beheer was relatief swak. By hoër waksladings (1.2 en 1.8 L per ton) was die maksimum residu-limiet (MRL) oorskry. Wakslaagbehandelings het tot 'n beduidende verlaging van koueskade gelei, en TBZ het 'n toevoegende effek gehad. Doopbehandelings het in die algeheel beter beheer gewys, maar wakslaagbehandelings het beter voorkomende beheer en laer

vlakke van sporulasie tot gevolg gehad. Stortbehandeling was minder effektief as doopbehandelings. TBZ-bestande isolate kon nie beheer word nie.

Imazalil-bestandheid in *P. digitatum* is ‘n kwantitatiewe eienskap waarby verskeie gene betrokke is. Drie van die meer bekende IMZ bestandheidsgroepe in *P. digitatum* is voorheen gekarakteriseer as R1, R2 en R3. Die relatiewe voorkoms van hierdie bestandheidsgroepe in populasies was grootliks onbekend. Polimerase ketting reaksie (PKR) voorvoerders wat voorheen gepubliseer is om tussen hierdie groepe te onderskei, is gebruik om IMZ-bestande isolate van *P. digitatum* uit Argentinië, Australië, Chile, Ciprus, Israel, Spanje, Uruguay, die VSA en Suid-Afrika te klassifiseer. ‘n Totaal van 189 isolate het PKR-produkte opgelewer wat op ‘n elektroforese gel geskei en gevisualiseer kon word. Een-en-veertig isolate kon nie geklassifiseer word nie, wat daarop dui dat ander ongekarakteriseerde weerstandsgroepe betrokke mag wees by IMZ-bestandheid in *P. digitatum.* Isolate van die VSA het die grootste verskeidenheid bestandheidsgroepe gehad: 13.7% van 73 isolate was in die R1-groep geklassifiseer, 12.3% in die R2-groep, 56.2% in die R3-groep en 17.8% was ongeklassifiseerd. Uit die 67 isolate van Chile, was 1.5% in die R1-groep, 76.1% was in die R3-groep en 22.4% was ongeklassifiseerd. Die 90 isolate van ander lande was almal in die R3-groep geklassifiseer (83.3%) of ongeklassifiseerd (16.7%). Omdat die R3-groep mees algemeen voorgekom het, kon die R3-geen as teikengeen gebruik word vir die ontwikkeling van ‘n kwantitatiewe PKR. Petri-bakkie toetse om IMZ-bestandheid te kwantifiseer was onbetroubaar en kon nie as ‘n vergelykingsmetode van die kwantitatiewe PKR gebruik word nie. Laasgenoemde is geoptimiseer vir miselium-DNA, maar was nie sensitief genoeg vir kwantifisering van spore uit die pakhuisomgewing nie.

ACKNOWLEDGEMENTS

I acknowledge with gratitude the following people and institutions:

Dr. Paul Fourie for guidance and providing countless opportunities for the acquirement of scientific skills

Arno Erasmus for his mentorship and advice

Dr. Adéle McLeod for providing advice on the molecular aspect of this study on such short notice

Dr. Paul Cronjé for insightful comments and suggestions on the chilling injury aspect of this study

Ilze Beukes and Lindy Rose for guidance on the real-time PCR work

Gideon Van Zyl for assisting with the image analysis of photos

Geert de Wever (Janssen PMP, Belgium) for supplying the international collection of *Penicillium digitatum* isolates

Prof. Karin Jacobs for supplying the other *Penicillium* spp. isolates used in this study

Prof. Megan Dewdney for constructive comments regarding real-time PCR

Elveresha Davids and Michelle Leibrandt for technical assistance

Amelia Kellerman for proofreading and editing my thesis

ICA Chemicals and John Bean Technologies for providing fungicides and wax

Citrus Research International, Technology and Human Resources for Industry Programme (THRIP) and National Research Foundation (NRF), and Citrus Academy for financial support

Friends at the department of Plant Pathology and other friends for friendship and encouragement

My parents and sister for their love, encouragement and interest in my studies

CONTENT

CHAPTER 1

EFFICACY OF THIABENDAZOLE APPLICATIONS TO CONTROL GREEN MOULD AND CHILLING INJURY, AND THIABENDAZOLE AND IMAZALIL RESISTANCE IN *PENICILLIUM DIGITATUM*

1. The South African citrus industry

Citrus is a key foreign exchange commodity and has been grown in South Africa since 1654 (Obagwu and Korsten, 2003). South Africa exported approximately 1 316 343 pallets of fruit in 2011 (CGA, 2012), making this country the second largest exporter of citrus in the world, exceeded only by Spain (DAFF, 2010). Annual production for 2009 was about 1387 thousand tonnes. It is only exceeded by the vegetable and combined deciduous fruit industries as a fresh produce industry in South Africa and added a value of R5.8 billion to the South African agricultural industry in 2008/9. The industry has grown both in production and in export quantities over the past ten years (DAFF, 2010), and employs a large proportion of the South African population (Obagwu and Korsten, 2003). Major export destinations for South African citrus include Europe, the USA, Korea and Japan (CGA, 2012). The amount of time from harvest until the citrus fruit reach the market ranges from 8 to 10 weeks (Pelser, 1977). During this time, postharvest diseases can cause significant losses. Heitmann stated that losses due to postharvest diseases can be as much as 6% of the total fruit exported from South Africa (Auret, 2000). Costs involved in postharvest diseases are not only for decayed fruit, but also for repacking undamaged fruit into new containers (Smilanick *et al.*, 2006b).

South Africa produces mostly Valencia and navel oranges, as well as grapefruit, soft citrus and lemons. Southern Africa has 17 different citrus-growing regions with unique climatic conditions (CGA 2012). Production is mainly in the Western Cape, Limpopo, Mpumalanga, Eastern Cape and Kwa-Zulu Natal provinces and, to a lesser extent, in the Northern Cape province, Zimbabwe, Swaziland and Mozambique (DAFF, 2010; CGA 2012). When exporting citrus, exporters may meet with barriers to trade that include sanitary and phytosanitary (SPS) risks, as well as specifications of quality and maximum residue levels (MRLs). One SPS-risk imposed by the European Union (EU) is citrus black spot (CBS) and European importers currently have a zero-percent tolerance to CBS. However, opposed to this, importers are requiring increasingly lower MRLs of fungicides used to control diseases. Another SPS-risk from South African citrus is false codling moth (FCM) infestation. Certain importers require cold treatment of South African citrus before accepting it into the market. Increasing competition from other Southern hemisphere citrus growers such as Chile, Brazil and Argentina (DAFF, 2010; Kotze, 1981) necessitates the overcoming of these trade barriers in innovative ways.

2. Citrus green mould

A large variety of postharvest diseases exist on citrus. They are mainly of fungal origin and include diseases such as *Alternaria* rot, anthracnose, *Aspergillus* rot, brown rot, cottony rot, *Dothiorella* rot, grey mould, blue mould, green mould, whisker mould, *Pleospora* rot, sour rot, stem end rots and *Trichoderma* rot (Timmer *et al.,* 2000). Latent pathogens causing significant problems in South Africa are *Diplodia* stem-end rot, anthracnose rot, *Phomopsis* stem-end rot, *Alternaria* rot, and *Phytophthora* brown rot (Keith Lesar, pers. comm.).However, the diseases causing the most economic losses in South Africa are green mould, blue mould and sour rot (Keith Lesar, pers. comm.), of which green mould, caused by *Penicillium digitatum* (Pers.:Fr) Sacc. Wehmer, is the most prevalent and economically important. Losses of up to R15 per carton can occur when citrus needs to be repacked at the port of destination due to green mould spoilage (Clint MacAleer, pers. comm.).

Penicillium spp. is classified in the phylum Ascomycota, in the class Plectomycetes, and order *Eurotiales* (Lawrence, 2008). The name *Penicillium* was first used as the genus name by Link in 1809. The Latin word 'penicillus' means brush and describes the morphology of the conidiophores. Not all species produce perithecia (Raper and Thom, 1949). Asexual reproduction happens through blastic-phialidic conidiogenesis, which leads to the production of large numbers of dry conidia (Kendrick, 1992). *Penicillium* spp. are frequently the cause of postharvest losses of fruit and vegetables. They cause up to 90% of postharvest losses of food from plant origin. Infection may already take place in the field. *Penicillium* spp. are wound pathogens and enter through wounds on the fruit. However, once infected, fruit may infect adjacent healthy fruit without wounds (Agrios, 2005). This is not frequently observed with *P. digitatum* on citrus, but more with *P. italicum* (Barmore and Brown, 1982). Sporulating fruit may cause soilage of healthy fruit in their vicinity, which makes cleaning and repacking healthy fruit necessary (Brown *et al.*, 1988). Typical symptoms are water-soaked lesions which enlarge quickly and white mycelia start growing on them. These may sporulate and produce a green or blue colour. A colony of *Penicillium* with a diameter of 2.5 cm may produce up to 4×10^8 spores (Kendrick, 1992). The fungi need moisture to form a mould. In dry conditions fruit do not rot, or, if already decayed, it will form mummies. *Penicillium* spp. produce several mycotoxins that are harmful to humans, e.g. patulin (Agrios, 2005). It is known that *Penicillium* spores may cause acute or chronic allergy symptoms in people whose work environment exposes them to high spore loads. Symptoms include chills and fever, or, if the allergy becomes chronic, sufferers may develop emphysema (Kendrick, 1992).

Saccardo in 1880 found *Penicillium* spp. which causes green mould on citrus**,** and named it *Penicillium digitatum* (Pers.:Fr) Sacc. Wehmer in 1894 found that blue mould is caused by *Penicillium italicum* Wehmer (Raper, 1949). Ninety percent of citrus fruit decay is due to *P. digitatum* infection (Eckert and Eaks, 1989), which is present in all citrus growing areas (Brown *et al.*, 1988). *Penicillium*

digitatum is only pathogenic on citrus, although it has been able to infect apples under certain conditions for a limited period of time (Buron-Moles *et al.,* 2012).

Penicillium digitatum's morphology is not typical to the *Penicillium* genus. It grows fast on malt extract agar (MEA) and produces olive green spores. The conidiophores are described by Samson *et al.* (2002) as being "irregularly branched, consisting of short stipes and branches terminating in whorls of 3-6 phialides, smooth walled". The conidia may have an elliptical or cylindrical shape, with an olive-green colour. *Penicillium digitatum* have biverticillate conidiophores and produce tryptoquialanines, a compound produced by only one other *Penicillium* species, *i.e. Penicillium aethiopicum* (Samson *et al.*, 2002; Gao *et al.,* 2011). *Penicillium digitatum* conidium formation is characterized by the conidia having a cylindrical shape for quite some time before it changes to an elliptical shape during maturation (Raper and Thom, 1949). *Penicillium digitatum* produces the enzyme pectin transeliminase, which macerates the citrus peel and enables it to cause decay (Bush and Codner, 1968). The acidification of its environment by enzymes such as polygalacturonase is an important virulence factor (Prusky *et al.,* 2004).

Penicillium digitatum survives on orchard debris and produce airborne spores that infect wounded and split fruit in the orchard (Brown *et al.,* 1988). Hough (1969) found that wounds in the oil glands of the fruit skin are more susceptible to decay than wounds in other peel tissue. However, deep (4 mm) penetrating wounds in tissue other than oil glands also led to significant decay. Variables such as peel thickness, region of fruit that was inoculated and the position of the fruit on the tree, did not influence the rate of decay in his study. Citrus cultivars may differ in susceptibility, e.g. lemons develop decay easier than grapefruit. Bates (1936) suggested that this is due to the fact that grapefruit's oil glands are few and are situated greater distances apart than lemons. Fruit that was inoculated on the tree developed latent infections, which developed symptoms during storage, but sporulation was less than on fruit that was inoculated postharvest (Hough, 1969).

In practice, wounds made by fruit flies and moths ovipositing on the fruit may be an important entrance for *P. digitatum* (Loest & Roth, 1963; Roth, 1967; Pelser, 1977). Fruit flies' ovipositors are frequently covered with *P. digitatum* spores (Loest and Roth, 1963).

The highest rate of decay is at temperatures of 15 – 29°C (Plaza *et al.,* 2003). Conidia can survive for up to 42 days at ambient conditions, and are shorter lived in humid conditions (Smilanick and Mansour, 2007).

3. Chilling injury

Cold storage is required by certain citrus importing countries as a quarantine measure against FCM and fruit fly infestation. Fruit from South Africa exported to China and the USA is kept at -0.6°C for 22 days (Hordijk *et al.,* 2013).

Chilling injury is a physiological disorder that reduces the quality of citrus fruit stored at 12°C or below (Eckert and Eaks, 1989). Chilling injury leads to a loss of cell membrane integrity (Marangoni *et al.,* 1996), and leaking of oil gland contents into adjacent parenchyma cells in the fruit rind. This leads to browning of the skin (Underhill *et al.*, 1995).

Chilling injury symptoms in citrus can vary between pitting, browning of the peel, scalding, sunken lesions and staining (Reuther *et al.,* 1989). Thiabendazole is known to protect citrus against chilling injury (CI) (McDonald *et al.,* 2011). Various factors improve the efficacy of TBZ in preventing chilling injury. Thiabendazole at lower doses in hot water is more effective against chilling injury than higher doses in cold water (Schirra *et al.*, 1998). Rodov *et al*. (1995) found that adding TBZ to the hot water led to injury of the fruit and nullified the CI reduction effect. They suggest that this injury may be prevented by optimizing the temperature and exposure time. The mechanism of CI reduction by TBZ is thought to be the delayed senescence of peel tissue, according to Schiffman-Nadel (Lindhout, 2007).

Another method of CI reduction is heat treatment where fruit is kept at 37°C for 3 days before cold storage, called curing. This releases more enzymes related to CI protection (Sala and Lafuente, 1999; Sapitnitskaya *et al.*, 2006). Heat treatments at higher temperatures for shorter exposure times (e.g. hot water dips) are more desirable because of reduced fruit weight loss and fruit have a more attractive looking peel (Porat *et al*., 2000; Fallik, 2004). Rodov *et al*. (1995) found that dipping citrus fruit in hot water (53°C for 2 min) led to reduction in CI. Hot water rinsing and brushing led to improved quality of various citrus cultivars (Fallik, 2004). Dipping fruit in methyl jasmonate (10 µM) for 30 s also reduces chilling injury (Droby *et al.,* 1999). Applying wax to fruit protects them against CI (Davis and Smoot, 1960; Wild, 1993), and improves internal quality (Salajeqe *et al.,* 2007).

4. Citrus as the host plant

Four main types of citrus are grown in South Africa: Sweet oranges (*Citrus sinensis* (L.) Osbeck), grapefruit (*Citrus paradisi* Macfadyen), lemons (*Citrus limon* (L.) Burm. f.) and soft citrus (*Citrus reticulata* Blanco) (DAFF, 2010). Different citrus types have different susceptibility to *P. digitatum*. Valencia oranges seem to be less susceptible to green mould than other types (Chalutz *et al*., 1985; Erasmus *et al*., 2011; Njombolwana *et al.*, 2013). Lemons were found to be more

susceptible than grapefruit to green mould by Hough (1969). The kind of rootstock on which navel and Valencia oranges and grapefruit were grown significantly influenced the amount of postharvest decay (McDonald and Wutscher, 1974; Ritenour *et al.,* 2004). For navel oranges different rootstocks lead to differences of 3% or more in the amount of decay observed. Season and storage temperature had an effect on the significance of results for Valencia oranges, but lead to a difference of 13% or more in the amount of decay observed. Unfortunately the study did not differentiate between types of decay observed, although mentioning that it was mainly green mould.

Grapefruit have a higher susceptibility to CI than lemons, which in turn is more susceptible than Valencia oranges (Chalutz *et al*., 1985). Schirra *et al.* (1998) found that Tarocco oranges harvested later in the season developed less CI than early season fruit. Schirra *et al.* (1998) also found that fruit maturity influenced the amount of TBZ residue loaded on oranges, and Liebenberg (2011) found that different fruit batches influence residue loading. The kind of rootstock used influenced postharvest rind quality in mandarin fruit (Cronjé, 2013).

Citrus fruit may contain the following preformed defence molecules (varying between citrus type) in their peel against fungal infection: citral, limettin, 5-geranoxy-7-methanoxycoumarin, and isopimpinellin (Ben-Yehoshua *et al.,* 1992). Induced defence includes the production of scoparone (Ben-Yehoshua *et al.,* 1992). Scoparone had a higher fungitoxic effect on *P. digitatum* than any of the preformed defence compounds, and was also elicited by heat and ultra-violet (UV) light treatment. It is produced in varying amounts in different citrus types (Ben-Yehoshua *et al.,* 1992).Green fruit is less susceptible than mature fruit to green mould (Schiffman-Nadel *et al.*, 1975). This is linked to the amount of certain phytoalexins produced in higher amounts by green fruit in response to infection compared to mature fruit. *P. digitatum* was also shown to be less virulent in green fruit (Afek *et al.,* 1999).The pH of green fruit rinds are higher than mature fruit, and inhibits spore germination (Smilanick *et al.,* 2005).

5. Methods of green mould control

5.1. Sanitation and prevention of wounds

Damaging the peel of fruit during harvest causes wounds that are entry points for *P. digitatum.* This should be avoided by educating pickers, and implementing general careful handling, good roads for transport of fruit, cleaning and repairing of bins in which fruit are packed and insect control (Lesar, 2006). Orchard sanitation including the removal of infected fruit and skirting of trees are highly effective methods (Hough, 1970). The infection cycle of *P. digitatum* is quite fast and occurs repeatedly in the packhouse in one season. This can lead to a build-up of inoculum if the packhouse is not cleaned thoroughly. Care should be taken that air currents do not carry spores from the site where fruit is tipped onto the packline further into the packhouse. This can be achieved by separating the tip site from the rest of the line, or by using extractor fans. High levels of spores in the packhouse favours fungicide resistance development and sanitation should therefore be practiced alongside chemical control (Hough, 1970; Bancroft *et al.,* 1984; Brown *et al.,* 1988). Putting fruit through a packline can cause bruising that can serve as entry points for *P. digitatum* (Skaria *et al.,* 2003). Fischer *et al.* (2009) quantified airborne spores in packhouses on PDA plates opened for 2 minutes at a time and found spore loads of 12.3 – 46.3 cfu/plate with the predominant fungal genera being *Cladosporium* and *Penicillium* spp. Wild and Eckert (1982) found that the exudates produced by benzimidazole-sensitive isolates of *P. digitatum* enhanced the virulence of benzimidazole resistant isolates. They suggest that packhouse sanitation is very important to reduce both sensitive and resistant spores.

Chlorine is commonly used to sanitise fruit and packhouse surfaces. Smilanick (2002) showed that efficacy of chlorine is pH, temperature and exposure-time dependent when used to kill green mould spores. Didecyl dimethyl ammonium chloride can also be used as a sanitizer for packhouse surfaces, and is also effective in sanitizing fruit by preharvest sprays (Rheinländer and Fullerton, 2007), although the latter practice is not recommended in South Africa(Arno Erasmus, pers. comm.).

5.2. Physical methods

Ultra-Violet (UV) light treatment of lemons leads to production of scoparone, an antifungal compound active against *P. digitatum* (Ben-Yehoshoua *et al.,* 1992). The efficacy of UV light is dependent on fruit maturity, seasonality and storage temperature (Droby *et al.,* 2007). The implementation of a UV light in a citrus packhouse is difficult because fruit will have to be rotated so that their whole area is exposed to UV for a long enough time. In certain fruit types, 0.8 s was long enough, but sometimes 2.9 s was required at a dose of 2.7 $W.m^{-2}$ (Droby *et al.,* 1993), but at lower doses, up to 1.75 minutes are needed (Stevens *et al.,* 1997). Application of benomyl was more effective than UV-C light treatments in preventing green mould on tangerines, but a combination of

UV light and fungicides or a biocontrol agent (*Debaryomyces hansenii*) produced superior results (Stevens *et al.,* 1997). There are still a lot of obstacles to its implementation in commercial packhouses (Palou *et al.*, 2008), e.g. phytotoxicity problems (Kinay *et al.,* 2005).

Irradiation at high enough doses (>1000 Gy) to kill *Penicillium* spores on fruit is not accepted by the public, although it has been shown as a very effective treatment (Palou *et al.,* 2008; Patil, 2004). Also, some adverse effects on the fruit rind may occur (Miller, 1996).

Curing, where fruit is exposed to hot air for a few days is known to lower green mould incidence (Palou, 2013). It is unfortunately costly and can lead to damaged fruit rinds. Degreening can be seen as a form of curing since it happens at temperatures of 29°C (Palou *et al.,* 2008), but the accompanying ethylene treatment may lessen its efficacy by stimulating green mould development (Schiffman-Nadel *et al.*, 1975). Some citrus growing regions such as Spain and Western USA degreen fruit at 20 – 22°C, which is conducive to green mould development (Joseph Smilanick, pers. comm.).

Dipping fruit in hot water for 2-5 min at 45 – 55°C or brushing hot water over fruit is also very effective at curing existing infections of green mould, but since there is no protective or long lasting action and the possibility of phytotoxicity, it is not done at most packhouses (Palou *et al.,* 2008).

Cold storage (3 - 5°C) is a common practice in the citrus export chain, as is cold-sterilisation treatment for insect infestation (-0.6°C, 22 days). The low temperature is fungistatic on *P. digitatum* and therefore used in combination with fungicides (Palou *et al.,* 2008).

Controlled atmosphere, where fruit is kept at 0 – 5% O_2 and 0 – 10% CO_2 showed conflicting results by different studies (Palou *et al.,* 2008).

Ozone fumigation can decrease the viability of *P. digitatum* conidia in water (Iseki *et al.,* 2010); however, it was not found to confer practical control of green mould during shipment, except for delaying the development of disease and inhibiting sporulation (Palou *et al.,* 2001). The efficacy of ozone treatment during shipment depends strongly on the way fruit was packed (Palou *et al.,* 2003). Dipping fruit into ozonated water did not cure infections of 24 h old (Smilanick *et al.,* 2002). Ozone seems to hold more promise for other fruit crops and pathogens, for instance blackberries and strawberries (Barth *et al.*, 1995; Nadas *et al.,* 2003).

5.3. Biological control

Currently, there is increasing concern about the unrestrained use of chemical pesticides. They have adverse effects such as toxicity on the environment, the plant and the consumer (Gerhardson, 2002). Pests that become resistant to pesticides are also a major problem. Large amounts of chemicals are used and cause damage to a wide range of resources while only targeting one or a few pathogens (Whipps, 1997).

Microbiological control organisms have different mechanisms of action to control plant disease, including competition, antibiosis, parasitism or predation, and inducing plant resistance (Larkin *et al.,* 1998). Biological control is an attractive potential control method for plant diseases because fungicides are increasingly undesirable to consumers, and pathogens may develop resistance to them (Compant *et al.,* 2005). *Candida oleophila*, a biocontrol yeast registered in Israel as Aspire®, was found to control green mould as a drench application, but control was variable. Also, the existing chemical regime showed better control (Droby *et al.,* 1998). Aspire's efficacy was dependent on the type and age of wound (Brown *et al.,* 2000). *Pseudomonas cepacia* controlled *P. digitatum* infections younger than 12 hours by a combination of antibiotic production and other modes of action (Smilanick and Denis-Arrue, 1992). Interestingly, an avirulent strain of *Galactomyces citri-aurantii,* an anamorph of the causal agent of citrus sour rot, was patented as a biocontrol agent for green mould in California (Eayre *et al.,* 2003). Biofumigation with *Muscodor albus*, an ascomycete that produces volatile compounds was effective in degreening rooms against green mould (Mercier and Smilanick, 2005). It was also found that frequently, a combination of biocontrol organisms with different mechanisms of action applied to plants were more effective than one species alone (Larkin *et al.,* 1998). Biological control has not been implemented in packhouses because of the variability in level of control obtained from biocontrol organisms, and also as a result of the difficulty in applying them in the packhouse and in developing practical formulations for them (Mari *et al.,* 2007). In packhouses where fungicide mixtures were used for longer periods and then pasteurised or chemically sanitised to remove foodborne pathogens, the biocontrol agent will also be removed by these practices and have to be reapplied (Joseph Smilanick, pers. comm.). Nonetheless, biocontrol shows great promise in enabling packhouses to administer lower doses of fungicides, especially as a protective control treatment (Moretto *et al.,* 2013).

5.4. Alternative chemicals

GRAS (Generally Regarded As Safe) compounds such as sodium carbonate, sodium bicarbonate (SBC), potassium bicarbonate and ammonium bicarbonate are known to inhibit mycelial growth of *P. digitatum*, but when spores are removed from these chemical suspensions, they are able to germinate (Smilanick *et al*., 1999). Lanza *et al.* (2002) found that many alternative chemicals

do not have a curative effect and also not a long lasting residue, and is best used in combination with conventional fungicides. It was found that sodium bicarbonate was the most promising compound for practical application in the packhouse (Smilanick *et al*., 1999), but it can cause moisture loss of fruit during storage (Schirra *et al*., 2008). GRAS compounds do not have a very long lasting effect (Palou *et al.,* 2002).

Many phenolic compounds show promise as sanitisers and curative treatments for green mould, for example, pomegranate peel extract was aerosolised and used as a sanitizing agent by Tayel *et al.* (2009). Other studies have found medicinal plant extracts and garlic has potential in controlling green mould (Khilare and Gangawane, 1997; Obagwu and Korsten, 2003).

5.5. Chemical control

In Western agriculture, insects and diseases on commercial crops are controlled mostly by chemical pesticides (Gerhardson, 2002). It is mostly supplemented by cultural control. This is also true for postharvest diseases (Korsten, 2006).

Thiabendazole and imazalil are the most commonly used postharvest green mould fungicides (Dodd, 2010). Azoxystrobin, fludioxonil, and pyrimethanil are new fungicides that are active against *P. digitatum* strains that are resistant to TBZ and IMZ. These fungicides have not been registered for use on citrus in some importing countries (Schirra *et al.*, 2008).

Consumers are increasingly concerned about synthetic chemicals used in agriculture and are demanding lower use of these chemicals (Korsten, 2006; Mari *et al.,* 2007). Specifically the US EPA has identified TBZ and IMZ as potentially carcinogenic fungicides, and therefore, the demand for lower MRLs of these fungicides are rising (US EPA, 2002; US EPA, 2005; Zhang, 2007).

Optimal application of these fungicides is of paramount importance to prevent the development of resistance in fungal populations, since there are only a few fungicides available. Also, in order to meet the consumer's demands of lower MRLs, the fungicides need to be applied in a manner that maximises its efficacy.

5.5.1. Thiabendazole

Thiabendazole (4-(1*H*-1,3-benzodiazol-2-yl)-1,3-thiazole) has been used for postharvest treatment of citrus since 1962 in the USA (Dave *et al.*, 1980). TBZ is a systemic fungicide with a broad range of action that can be applied to many different crops (Marsh *et al.,* 1977). It does not lead to phytotoxicity and has a long residual activity (Ladaniya, 2008). TBZ inhibits fungal cell division, and is therefore like most fungicides, fungistatic (Clemons and Sisler, 1971). At high concentrations, TBZ also inhibits several enzymes involved in oxygen consumption, respiration, protein and nucleic acid synthesis and nutrient uptake (Allen and Gottlieb, 1970). TBZ has a pKa value of 4.6 (Koner *et al.,* 2011) and is maximally soluble in water at pH 2.5 and can form stable chelates with metals (Brown *et*

al., 1961). In fungicide dip tanks of neutral pH and in water-based wax, it tends to precipitate (McCornack, 1970; Ladaniya, 2008). On citrus it is used to control the postharvest diseases green mould, blue mould, Diplodia stem-end rot and anthracnose (Wardowski and Brown, 1991). Thiabendazole can be used curatively (Schirra *et al*., 2008) and protectively (Brown, 1977) against *P. digitatum*, and also controls its sporulation (Ladaniya, 2008). Thiabendazole is a benzimidazole fungicide related to benomyl, carbendazim and thiophanate-methyl, and these compounds may be applied to citrus orchards to control various diseases including citrus black spot (Kellerman and Kotze, 1979; Ladaniya, 2008). Thiophanate methyl, a fungicide in the same chemical group as TBZ, reduced the postharvest incidence of green mould when applied preharvest by Smilanick *et al.* (2006a). They warned that frequent preharvest application of benzimidazole fungicides may lead to resistant isolates in orchards, to which postharvest application of TBZ in the packhouse will be ineffective.

Benomyl gave better control than TBZ against green mould and stem-end rot, but gave the fruit peel an unattractive colour (Wild *et al.,* 1975).

5.5.1.1. Thiabendazole residue loading

The maximum residue level (MRL) of thiabendazole on citrus according to *Codex alimentarius* is 7 $\mu g.g^{-1}$, but in EU member countries it is 5 $\mu g.g^{-1}$ (FAO, 2012). Residue loading of TBZ can be increased by managing several physical factors in the packhouse. Cabras *et al.* (1999) found that washing fruit under cold water removed 90% of TBZ residues. Eckert and Kolbezen (1977) and Brown (1984) also found that TBZ does not penetrate the fruit rind. The addition of sodium bicarbonate (SBC) to an aqueous TBZ suspension caused lower or unchanged residue levels of TBZ on fruit, but lead to better penetration of the fungicide into the fruit skin (Schirra *et al.,* 2008). This is probably linked to the observation of Smilanick *et al.* (2008) that an increased pH makes TBZ less soluble in the bath. The pH of a TBZ and SBC suspension increased over 24 hours (D'Aquino *et al.,* 2013). Opposed to this, Wardowski *et al.* (1974) found that an increased pH led to increased TBZ residues on fruit. D'Aquino *et al.* (2013) found a doubling in the TBZ residue loaded onto fruit if SBC was added, but this declined during storage for 12 days at 17°C to a similar level than on fruit dipped in TBZ only. Smilanick *et al.* (2008) found that the TBZ residue on fruit was not increased by addition of potassium sorbate (KS). TBZ applied in hot water increased its uptake by the fruit (Smilanick *et al*., 1997), specifically into the fruit cuticle (Schirra *et al.,* 2008), and higher residues were loaded with hot TBZ suspensions than cold ones in a more recent study (Palma *et al.,* 2013). Fruit maturity can play a role: over-mature fruit (harvested April to June in Northern hemisphere) contained more TBZ than fruit picked in November to January (Schirra, 2000). Smilanick *et al.* (2006a) found a linear relationship between TBZ concentration and TBZ residue on lemon fruit. At 1000 $\mu g.mL^{-1}$ TBZ residues of approximately 3 – 4 $\mu g.g^{-1}$ TBZ was loaded at 24°C and 5 – 6 $\mu g.g^{-1}$ TBZ at 41°C. Increasing the time

that fruit stays immersed did not have any effect in the study of Smilanick *et al.* (2006a). Similar residue loading was observed by Palma *et al.* (2013) when fruit was dipped in 1000 $\mu g.g^{-1}$ TBZ at 20°C and 300 $\mu g.g^{-1}$ TBZ at 53°C, but the residues of the heat treated fruit were more persistent during storage. They suggested that the heat caused the epicuticular wax on the fruit to melt and encapsulate the fungicide. El-Tobshy *et al.* (1982) loaded residues of 1.3, 1.7 and 3.3 $\mu g.g^{-1}$ on navel oranges with TBZ concentrations of 4000, 6000 and 8000 $\mu g.mL^{-1}$ respectively, applied as a dip followed by waxing. Longer dip times do not lead to increased residue loading, but led to better uptake of fungicide into the fruit skin at high temperatures (Schirra *et al.,* 2008). Mixing TBZ with other fungicides can influence its residue loading, for example, TBZ mixed with fludioxonil led to higher TBZ residues than TBZ alone (D'Aquino *et al.,* 2013). TBZ residues on the fruit did not change significantly during cold storage in the studies of Hayward and McCornack, 1971 and Cabras *et al.* (1999). A small decrease in TBZ residues during storage of Tarocco oranges was observed by Palma *et al.* (2013).

TBZ can also be applied in wax coatings. This is a popular application method because it reduces the cost of fungicide application (Gutter, 1970). Hayward and McCornack (1971) found that TBZ in wax loads higher residues than TBZ in dip treatments. Gutter (1970) and Brown (1984) suggested that brushes may remove TBZ residue in the packhouse after dipping and that applying TBZ through wax might counteract this problem. El-Tobshy *et al.* (1982) loaded residues of 3.9, 5.1 and 6.7 $\mu g.g^{-1}$ on navel oranges with TBZ concentrations of 4000, 6000 and 8000 $\mu g.mL^{-1}$ in wax coatings, respectively. Eckert and Kolbezen (1983) loaded 5.16 $\mu g.g^{-1}$ TBZ on fruit waxed with 4280 $\mu g.mL^{-1}$ TBZ amended wax.

TBZ applied as a non-recovery aqueous spray resulted in very low TBZ residues (< 1 $\mu g.g^{-1}$) (Hayward and McCornack, 1971).

5.5.1.2. Green mould control by thiabendazole

Schirra *et al.* (2008) stated that the location of the fungicide is more important than the amount of residue present on the fruit for control of resistant isolates. Smilanick *et al.* (2006a) and Schirra *et al.* (2008) found that applying TBZ as a dip at higher temperatures together with sodium bicarbonate (SBC) effectively controlled a TBZ resistant isolate of *P. digitatum*, and that a lower concentration of TBZ was needed to control sensitive isolates with these modifications. Adding more than 0.5% SBC to the mixture caused moisture loss in the fruit (Schirra *et al.,* 2008); the extent of water loss above this level has not been shown. The addition of SBC led to an increase of pH of the TBZ suspension over time, and had a synergistic effect with TBZ in controlling green mould curatively (D'Aquino *et al.,* 2013). Smilanick *et al*. (2005) found that SBC can control green mould even at a neutral pH. Smilanick *et al.* (2008) found that the addition of 0.5% potassium sorbate (KS) to TBZ

formulations increased the efficacy of TBZ to both resistant and sensitive isolates of *P. digitatum*. Potassium sorbate performed optimally at a pH of 4 – 6, and moderately enhanced TBZ's efficacy as a drench and a dip treatment. Potassium sorbate is easier to dispose of than SBC because it does not contain sodium. Wild (1987) found that the addition of 5% KS to TBZ did not control resistant isolates. Adding chlorine at 200 $\mu g.mL^{-1}$ to the TBZ suspension also improved green mould control (Smilanick *et al.,* 2006a). Gutter (1970) stated that high TBZ concentrations (2000 $\mu g.mL^{-1}$) applied as a dip inhibited sporulation.

TBZ in wax was less effective than the aqueous suspension in terms of green mould control (Gutter, 1970; Eckert and Kolbezen 1977; Brown, 1984). El-Tobshy *et al.* (1982) also found that TBZ dips were more effective in controlling sporulation than TBZ waxes of the same concentration, even though TBZ waxes loaded a higher TBZ residue on the fruit. Gutter (1970) suggested that the reduced costs of fungicide application through wax should be compared to the cost in postharvest losses before deciding on application method of TBZ. He also suggested using higher concentrations of TBZ in wax, but warns against MRL limitations. He found that higher TBZ concentrations in wax resulted in sporulation inhibition. Nelson (1984) found that adding potassium sorbate to TBZ- amended wax, controlled resistant isolates of *P. digitatum,* but dipping fruit in an aqueous suspension of potassium sorbate followed by TBZ-amended wax, gave the best control. Sporulation inhibition was generally achieved by treating citrus fruit with TBZ-amended wax (Hall *et al.,* 1978; El-Tobshy *et al.,* 1982; Eckert and Kolbezen, 1983). Youssef *et al*. (2012) found that application of non-amended wax led to a higher incidence of decay.

Zhang and Swingle (2005) found that drenching grapefruit with 500 $mg.L^{-1}$ TBZ had a curative action that could be improved if fruit was cured by hot air afterwards. Smilanick *et al.* (2006a) improved drenching efficacy by adding SBC and chlorine to the TBZ suspension, but increased temperature had no effect. They observed that dipping fruit in the same suspension was more effective than drenching.

5.5.1.3. Chilling injury control by thiabendazole

Thiabendazole is also known to have a beneficial effect on citrus fruit by protecting against chilling injury. Cold storage is required by certain citrus importing countries as a quarantine measure. Various factors improved the efficacy of TBZ in preventing chilling injury. Schirra *et al*. (1998) found that 200 $mg.L^{-1}$ TBZ at 50°C provided protection against chilling injury. TBZ at 1200 $mg.L^{-1}$ at 20°C also protected fruit against chilling injury, but not as much as 200 $mg.L^{-1}$ TBZ at 50°C. TBZ at 1000 $mg.L^{-1}$ at 53°C gave superior chilling injury protection than just hot water (MacDonald *et al*., 1991). The mechanism of CI reduction by TBZ is thought to be the delayed senescence of peel tissue, according to Schiffman-Nadel (Lindhout, 2007). Wax had an additive effect in reducing chilling injury compared

to TBZ alone, most likely due to inhibition of moisture loss and prevention of transpiration (Wild, 1993). Schirra *et al.* (2000) also found that dipping grapefruit in TBZ reduced chilling injury, while Dou (2004) found that carnauba wax reduced chilling injury on its own. Schiffman-Nadel *et al.* (1975) found that incorporating TBZ in wax was more beneficial than first dipping grapefruit in TBZ before waxing.

5.5.1.4. Thiabendazole resistance

The asexual reproduction cycle of *Penicillium* spp. predisposes them to the development of resistance to fungicides (Kendall and Hollomon, 1998), and because of the production of large numbers of haploid conidia, the effect of mutations on fungicide resistance is immediate (Georgopoulos and Skylakakis, 1986). Van Tuyl (1977) found that TBZ is a mutagenic compound to various fungi. TBZ is classified as a high risk for development of fungicide resistance (Brent and Hollomon, 1998). Green mould has been controlled effectively by TBZ, but problems with resistant isolates have arisen. Holmes and Eckert (1999) and Kinay *et al*. (2007) have found *P. digitatum* biotypes in packhouses that were resistant to TBZ, IMZ and ortho-phenylphenol (OPP) simultaneously. Opposed to this, they found that *P. digitatum* isolated from orchards were all sensitive to TBZ where benzimidazole fungicides were not sprayed as a preharvest treatment. Holmes and Eckert (1999) found that the level of resistance to TBZ in *P. digitatum* did not increase from 1986 to 1994. Kinay *et al*. (2007) also found that the EC_{50} level of TBZ resistant *P. digitatum* isolates stayed constant, while resistance levels to other fungicides varied. Lee *et al.* (2011) found that 37 out of 40 isolates collected in Taiwan were TBZ resistant. Sánchez-Torres and Tuset (2011) found 84% of isolates collected in Spain were TBZ-resistant, whereas Fischer *et al.* (2009) found that 25.9 to 51.9 % of packhouse isolates from 2 packhouses in Brazil were TBZ-resistant.

Georgopoulos and Skylakakis (1986) stated that in order for resistance to develop, a genetic basis for resistance must be present in the population, and development of resistance will then depend on the amount of selection pressure and the differing fitness levels of sensitive and resistant strains. Sanchez-Torres *et al.* (2011) and Lee *et al.* (2011) confirmed that TBZ resistance in *P. digitatum* is caused by a single mutation in the β-tubilin gene. Amino acid 200, phenylalanine, is substituted by tyrosine. At DNA-level, the nucleotide at position 992 of the gene coding for resistance, a thymine base was replaced by adenine (Schmidt *et al*., 2006). Georgopoulos and Skylakakis (1986) stated that if the cause of resistance to a fungicide is located in a single major gene, a sudden and total loss of control is possible. Thus, TBZ resistance in *P. digitatum* is qualitative and caused by a single gene and carries a great risk resulting in loss of control (Kanetis *et al.,* 2010). Van Tuyl (1977) did not find a loss in pathogenicity of *Penicillium expansum* isolates resistant to benzimidazoles, but slight differences in colony colour and lesion size were observed between

sensitive and resistant *P. digitatum* isolates in the study of Kinay *et al.* (2007). The fact that TBZ-resistance is linked to a mutation within the coding region of the target gene may explain the fact that TBZ-resistance is qualitative in a haploid pathogen such as *P. digitatum*, because TBZ is no longer able to bind to the target protein. This may lead to a sudden and complete loss of control should resistance occur in a packhouse.

5.5.2. Imazalil

Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-lH-imidazole) is currently the most dependable fungicide used to control green mould (Ladaniya, 2008). It is a sterol demethylation inhibitor (Hamamoto *et al.*, 2000), and is part of the azole fungicide group which inhibits ergosterol biosynthesis (Siegel & Ragsdale, 1978). More specifically, it inhibits the enzyme lanosterol 14α-demethylase, which is involved in the ergosterol biosynthetic pathway. The demethylation of the sterol molecule is essential in order for it to insert into the phospholipid bilayer of the cell membrane and it influences membrane fluidity (Gallay and De Kruijff, 1982; Rodriguez *et al.,* 1985). Ergosterol is a component of the fungal cell membrane, but not of other eukaryotes (Vanden Bosche, 1985). Azoles are fungistatic, but can become fungicidal when combined with other compounds that target different enzymes of the ergosterol biosynthesis pathway (Onyewu *et al*., 2003). The amino acid sequence for lanosterol 14α-demethylase is highly conserved, but there are a few key differences among fungal species (Joseph-Horne and Hollomon, 1997).

Imazalil leads to swollen conidia in *P. italicum*, and germ tube elongation is inhibited. The manner of deposition of β–1,3 and β-1,4 polysaccharides is unusual as well (Kerkenaar and Barug, 1984).

5.5.2.1. Imazalil resistance

Soon after the introduction of IMZ to control TBZ resistant isolates, resistance developed, and the number of resistant isolates kept increasing (Holmes & Eckert, 1999). Azole fungicides are classified as having a moderate risk of resistance development, and are being researched intensely in the medical field due to increasing problems with resistance (Brent and Hollomon, 1998). Sánchez-Torres and Tuset (2011) found that 77% of their *P. digitatum* isolates collected in Spain was IMZ-resistant. Holmes and Eckert (1999) found IMZ resistant isolates of *P. digitatum* in packhouses, but found that the level of resistance stayed constant over a 2-year period and suggested that this was due to the selection pressure in packhouses that stayed constant. They found that the EC_{50} levels of IMZ resistant isolates varied, depending on the age of inoculum used for the trial. Kinay *et al.* (2007) also found varying EC_{50} levels in IMZ resistant isolates. IMZ sensitive isolates of *P. digitatum* seem to be generally more fit than IMZ resistant isolates (Dave *et al.,* 1989), but Holmes and Eckert (1995) found some IMZ resistant isolates that were more fit than sensitive isolates. Van Tuyl (1977) did not

find lower virulence in IMZ-resistant isolates of several fungi compared to sensitive isolates. Zhu *et al.* (2006) found no difference in fitness between sensitive and resistant isolates. Kinay *et al.* (2007) found that in the absence of IMZ application, populations of 1:1 resistant to sensitive *P. digitatum* isolates reverted back to mostly sensitive isolates, but in some cases resistant isolates persisted. This was also found in earlier studies (Holmes and Eckert, 1995). A good indication of practical IMZ resistance in packhouses is the growth of isolates on PDA amended with 1 $mg.mL^{-1}$ IMZ (Perez *et al.,* 2011).

IMZ resistance is quantitative with multiple genes involved for several fungi, including *P. digitatum* (De Waard and Van Nistelrooy, 1990; Kanetis *et al.*, 2010). Three of the more prevalent IMZ resistance genotypes of *P. digitatum* have been characterised and termed R1, R2 and R3 (Sun *et al.*, 2011). These genotypes lead to the over-expression of genes coding for enzymes in the biochemical pathway that imazalil targets (Sánchez-Torres and Tuset, 2011). An insertion of a 126 bp four times repeated tandem repeat transcriptional enhancer into the promoter of CYP51A was named R1. This mechanism of resistance (tandem repeat) is thought to be only present in *P. digitatum* (Hamamoto *et al.*, 2000). R1-resistance was found in most of Hamamoto's isolates (Hamamoto *et al.*, 2000), but only in three isolates of Sánchez-Torres and Tuset (2011). The R2-resistance type is a 199 bp insertion in the CYP51A gene and it was not found to be homologous to any sequence in Genbank. The insertion occurs between nucleotides -55 and -56 (Ghosoph *et al.*, 2007). A 199 bp insertion in CYP51B gene's promoter was termed R3 (Sun *et al.*, 2011). R3-resistance is the most widespread type of IMZ resistance in China; 94% of resistant isolates had the R3-genotype. Increased expression of genes CYP51A, CYP51B and CYP51C (a third gene homologous to CYP51A and CYP51B) was observed in an IMZ sensitive isolate after treatment with IMZ (0.1ppm) (Sun *et al.,* 2011).

Mutations in the promoter and coding regions of PMR1 and PMR5 genes have been linked to IMZ resistance; these are ATP binding cassette (ABC)-transporter genes that are activated by the presence of toxicants and prevent accumulation of IMZ in the fungal cell (Sánchez-Torres and Tuset, 2011; Sun *et al.,* 2011). In other fungi, such as *Aspergillus nidulans,* treatment with IMZ leads to the increased expression of ABC-transporter genes (Del Sorbo *et al.,* 1997). ABC-transporter genes were shown in several fungi to confer both resistance to fungicides as well as promote pathogenicity and/or virulence in the host (Urban *et al.,* 1999; Andrade *et al.,* 2000; Schoonbeek *et al.*, 2001; Hayashi *et al.,* 2002). This might explain why some IMZ resistant isolates of *P. digitatum* were found to be more virulent than sensitive ones (Holmes and Eckert, 1995), therefore it is important to know the molecular base of resistance of an isolate to predict its effect on disease management. The quantitative nature of IMZ resistance is quite understandable since resistance is linked to either

overexpression of the target enzyme, or to exclusion of the fungicide by the cell due to overexpression of ABC- transporter genes. Therefore a gradual loss of control is expected.

The number of resistant isolates seems to vary between locations and time frames. Kinay *et al.* (2007) found an IMZ resistance frequency of 86% in California, while Zhu *et al.* (2006) only found 2% in China. Seventeen percent was found by Bus *et al.* (1991) in Europe. Fogliata *et al.* (2000) found only 5 isolates in Argentina resistant to IMZ, and only 3 of them was not controlled *in vivo* by 1000 $\mu g.ml^{-1}$ IMZ. Low frequencies (1.5 and 0.1%) of resistant isolates were found in two packinghouses in Brazil (Fischer *et al.,* 2009).

6. Quantification of pathogens and fungicide resistance

The number of spores in a packhouse has a big influence on disease control and the development of resistance (Wild and Eckert, 1982). Bancroft *et al.* (1984) used PDA plates amended with neopeptone, dichloran and TBZ to monitor TBZ resistance of *P. digitatum* in packhouses. Smilanick and Eckert (1986) improved this method by adding o-phenylanizole (OPA) and pentachloronitrobenzene (PCNB) to the media. Plates were exposed in packhouses for a certain amount of time and the number of colonies counted and compared to the number of colonies on plates not amended with TBZ to determine the amount of resistant spores. Exposed plate assays where plates were opened twice a week for an unknown amount of time did not give enough data to distinguish between packhouses' spore loads (Bancroft *et al.,* 1984). The authors suggest that spore loads in packhouses are too variable for short times.

Van Wyk (2011) suggested that molecular quantification methods are more reliable because PDA dishes dry out quickly and can only be used for short periods of time. Several pathogens have been quantified by real time PCR. Real time PCR assays are very specific and reliable and contamination is prevented (Chevaliez *et al.,* 2012). Bates and Taylor (2001) quantified *Pyrenophora teres* in barley seed and used scorpion amplified refractory mutation system (ARMS) primers to distinguish it from *Pyrenophora graminea* by a single nucleotide mismatch. Fraaije *et al.* (2002) quantified strobilurin resistant isolates of *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici* on wheat using SYBR green chemistry and allele-specific primers and were able to detect one resistant allele in 10 000. Michalecka *et al.* (2011) quantified strobilurin resistant *Venturia inequalis* from apple tree leaves using SYBR green chemistry. Haugland *et al.* (2004) quantified airborne fungal conidia using 65 different real time PCR assays. Schweigkofler *et al.* (2004) and Van Wyk *et al.* (2011) have quantified *Fusarium circinatum* spores from pine plantations using SYBR green chemistry. Boutigny *et al.* (2011) also quantified *Fusarium* pathogens from South African maize using the same method. TaqMan probes were used to quantify several different rotavirus genotypes by Gutiérrez-Aguirre *et al.* (2008).

Many real time PCR assays have been automated and are used in routine diagnostics; however, their upper limit of detection seems to present a problem in that frequently samples were found that contained substantially higher amounts of pathogens than the assay was able to detect (Chevaliez *et al.,* 2012). Some real time PCR assays have been unable to quantify all variants of a specific pathogen because of polymorphisms occurring at the annealing sites of primers and probes (Chevaliez *et al.,* 2012).

Another widely used method of quantifying and distinguishing between different genotypes of pathogens is high-resolution melting (HRM). The principle that a mixture of two different amplicons generates different melting curves from that of each individual amplicon was demonstrated by several studies (Wittwer *et al.,* 2003; Yeh *et al.,* 2004; Dobrowolski *et al.,* 2008). Further exploration of this method revealed that mixtures of different quantities of each amplicon led to different melting curves (Ganopoulos *et al.,* 2011; Madesis *et al.,* 2012), which can be distinguished from each other by calculating a genotype confidence percentage (Hewson *et al.,* 2009).

Next generation sequencing methods such as IonTorrent, HiSeq, MiSeq and pyrosequencing can also be used to quantify pathogens. The advantage of these methods is that less common variants of pathogens are also detected as opposed to using real time PCR assays. The disadvantages are that a large amount of data analysis is required and sequencing errors may occur (Chevaliez *et al.,* 2012).

7. Conclusion

Chemical control of green mould seems to be the most widely used and trusted method. It is clear that different fungicide application methods influence the fungicide's efficacy in controlling green mould. For TBZ, mostly the curative action in controlling green mould was investigated, especially following dip treatments. It is also clear that the method of TBZ application has a greater influence on its efficacy than the amount of residue loaded on fruit. Most studies concerning TBZ have been done before the last 10 years and none in the South African context, and little information is available concerning the current application specifications that is used for TBZ. This highlights the need to study optimal application of TBZ in South African packhouses.

Monitoring fungicide resistance is a fundamental practice in order to prolong the use of fungicides, as well as to indicate the efficacy of certain measures to prevent development of resistance. It can be anticipated that the frequency of TBZ- and IMZ-resistant *P. digitatum* isolates vary across time and locations. Most methods used to quantify fungicide resistance are time consuming. Although qPCR assays have been developed to detect certain genotypes of IMZ

resistance (Chen *et al.,* 2008), no molecular method has been published to quantify fungicide resistance in *P. digitatum* and only one method to quantify airborne spores of this species (Haugland *et al.,* 2004). Hence, the need to develop a reliable assay to monitor fungicide resistance in packhouses by quantifying the amount of resistant *P. digitatum* spores. Because IMZ-resistance has a polygenic nature, it presents a greater challenge in developing a molecular quantification tool than for instance strobulurin resistance, which is affected by a single mutation in a single major gene.

8. References

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CHAPTER 2

THIABENDAZOLE RESIDUE LOADING IN DIP, DRENCH AND WAX COATING APPLICATIONS TO CONTROL GREEN MOULD AND CHILLING INJURY ON CITRUS FRUIT

Abstract

Green mould (caused by *Penicillium digitatum*) is a major cause of postharvest losses in citrus. Residue loading of thiabendazole (TBZ) with application methods typically used in South African packhouses and green mould control was studied. TBZ was applied curatively and protectively in dip, drench and wax coating treatments and fruit were inoculated with a TBZ-sensitive or a TBZ-resistant isolate of *P. digitatum*. The dip treatments consisted of TBZ concentrations of 0 – 2000 $\mu g.mL^{-1}$; fruit were dipped for 60 s at 22°C at a pH of 7. Residues differed between fruit batches and ranged from 0.5 – 1.7 $\mu g.g^{-1}$ at 1000 $\mu g.mL^{-1}$ TBZ. Curative dip treatments almost completely controlled green mould (>96% at 1000 $\mu g.mL^{-1}$ TBZ). The residue level needed for 75% control ranged from 0.06 to 0.22 $\mu g.g^{-1}$ depending on citrus type. Protective treatments were unreliable and varied from 17 to 97.9% at 1000 $\mu g.mL^{-1}$ TBZ between fruit batches. Drench treatments consisted of exposure times of 30, 60 and 90 s with 1000 or 2000 $\mu g.mL^{-1}$ TBZ. Average TBZ residues were 2.14 $\mu g.g^{-1}$ for Clementine mandarin fruit and 3.50 $\mu g.g^{-1}$ for navel orange fruit. Green mould control on navel orange fruit resulted in 77 – 92%, 68 – 90% and 32 – 38% control for curative treatment after 6 and 24 h and protective treatments, respectively. On Clementine mandarin fruit it resulted in 66 – 84%, 34 – 56% and 9 – 17%, respectively. Wax with 4000 $\mu g.mL^{-1}$ TBZ was applied at 0.6, 1.2 and 1.8 L $wax.ton^{-1}$ fruit. Chilling injury was evaluated after fruit storage at -0.5°C for 40 days. Average TBZ residues loaded was 1.3, 1.3 and 2.7 $\mu g.g^{-1}$ at the recommended 1.2 $L.ton^{-1}$ for Satsuma mandarin, Clementine mandarin and Valencia orange fruit, respectively. Protective treatments showed lower infection levels (14 – 20%) than curative treatments (27 – 40%) for Valencia orange fruit. The same trend was observed with Satsuma (92 – 95% curative; 87 – 90% protective) and Clementine mandarin fruit (82 – 90% curative; 59 – 88% protective), but control was relatively poor. TBZ application in wax exceeded 5 $\mu g.g^{-1}$ at higher wax loads (1.2 and 1.8 L/ton). Wax treatments showed a significant reduction in chilling injury; TBZ had an additive effect. Dip treatments gave better overall control of green mould, but wax application showed a better protective treatment as well as lower sporulation incidence. Drench treatment was much less effective than dip treatments. TBZ resistant isolates could not be controlled.

1. Introduction

Thiabendazole (4-(1H-1,3-benzodiazol-2-yl)-1,3-thiazole; TBZ) is a benzimidazole fungicide and can be used curatively (Schirra *et al*., 2008) and protectively (Brown, 1977) against *Penicillium digitatum,* which causes green mould on citrus, and may lead to 90% of postharvest losses (Eckert and Eaks, 1989), and also controls its sporulation (Ladaniya, 2008). The maximum residue level (MRL) of TBZ on citrus according to *Codex alimentarius* is 7 $\mu g.g^{-1}$, but in European Union member countries it is 5 $\mu g.g^{-1}$ (FAO, 2012), although the consumer may impose even lower residue standards.

Postharvest TBZ treatment has also been shown to reduce chilling injury, a physiological disorder that reduces the quality of citrus fruit stored at 12°C or below (Eckert and Eaks, 1989). Certain importers require cold sterilisation treatment of South African citrus as a mitigating control measure against certain insect pests. For example, fruit from South Africa exported to China and the USA is kept at -0.6° C for 22 days, and these measures increase the risk of chilling injury (Paul Cronjé, pers. comm.).

Although imazalil (IMZ) is the preferred fungicide for control of green mould (Ladaniya, 2008; Erasmus *et al.,* 2011), IMZ resistance in *P. digitatum* populations have also been observed (Holmes and Eckert, 1999; Kinay *et al.,* 2007; Sánchez-Torres and Tuset, 2011), therefore sole reliance on IMZ is not advised.

There are several methods of applying TBZ to fruit, and different methods influence the efficacy of TBZ to control disease. Common methods in South Africa are drenching, dipping and wax coating with TBZ. Degreening conditions favour the development of green mould, therefore effective fungicide application in the postharvest drench is crucial (Smilanick *et al.,* 2006). Dipping fruit in an aqueous suspension of TBZ is not a popular application method because TBZ tends to precipitate (McCornack, 1970). Schirra *et al.* (2008) did extensive studies with TBZ in the dip tank, and stated that the location of the fungicide is more important than the amount of residue present on the fruit for control of *P. digitatum*. Their study found that different application parameters led to TBZ being loaded at different depths into the fruit skin, and this influenced the fungicide's bio-efficacy, even in controlling TBZ-resistant isolates. It is therefore important that the application of TBZ is optimal to ensure efficacy and prevent the development of resistance. Thiabendazole can also be applied in a wax coating, and this is a popular method because it saves costs in fungicide application (Gutter, 1970), and it loads higher residues on fruit (Hayward and McCornack, 1971), but it was shown to be less effective in controlling disease than other application methods (Brown, 1984).

Fruit can be treated before (protectively) or after (curatively) infection, and the method of fungicide application influences the efficacy of each treatment action differently. It was found that fungicides applied in a dip tank lead to better curative control (Smilanick *et al.,* 2006a; Dore *et al.,*

2009; Erasmus *et al.,* 2011; Erasmus *et al.,* 2013) and fungicides applied in a wax coating lead to better protective control (Waks *et al.,* 1985; Njombolwana *et al.,* 2013a). Wounds occur on fruit due to poor harvesting practices, and this is an entry point for *P. digitatum* spores from the orchard (Smilanick *et al.,* 2005). Wounds can also occur as a result of packline injuries (Skaria *et al.,* 2003), and the development of chilling injury and other rind conditions may be an entry point for spores found in packhouses (Brown and Eckert, 1988; Besri *et al.,* 1990; Jacobs *et al.,* 2010). The latter situation calls for effective protective control of green mould. Many studies have been done concerning the curative action of TBZ (Smilanick *et al.,* 2006b; Schirra *et al.,* 2008; Smilanick *et al.,* 2008; D'Aquino *et al.,* 2013), but relatively few concerning its protective action (Brown, 1977; El-Tobshy *et al.,* 1987).

TBZ resistance in the South African *P. digitatum* population was first observed in 1976 (Pelser, 1977), and the resistance spread rapidly (Dodd *et al.,* 2010). TBZ is classified as a high risk for development of fungicide resistance (Brent and Hollomon, 1998). TBZ resistance in *P. digitatum* is qualitative and caused by a single gene, and carries high risk in resulting in loss of control (Kanetis *et al.,* 2010).

Erasmus *et al.* (2011) did a survey to determine the most commonly used fungicide application methods and parameters in South African citrus packhouses. Most packhouses submerged fruit in a dip tank for about 48 s, and only 25 % dipped for longer than 60 s. The most common dip tank suspension temperature was 34°C, and the most common pH was 5.4. Most packhouses used the same dip tank solution for 5 days before preparing a fresh mixture (Erasmus *et al.,* 2011). The most common wax coating load at which fungicides are applied, is 1.2 L wax.ton^{-1}, whereas drench mixtures are usually applied at 250 L.bin^{-1} (Arno Erasmus, pers. comm.). No work has been done to evaluate TBZ efficacy under these conditions. The aim of this study therefore was to study TBZ residue loading and its efficacy in controlling green mould on citrus in three different applications: aqueous dip, wax coating and drench as commonly applied by South African citrus packhouses. Thiabendazole's efficacy in controlling a TBZ resistant isolate by means of these application methods, as well as reducing chilling injury when applied through a wax coating was also studied.

2. Materials and methods

2.1. Fruit

Satsuma and Clementine mandarin fruit (*Citrus reticulata* Blanco), and 'Washington' navel and 'Midknight' Valencia orange fruit (*Citrus sinensis* (L.) Osbeck) were obtained from packhouses in Franschhoek and Citrusdal. It was ensured that fruit were harvested at most 2 days before collection

and not treated with any post-harvest chemicals. The 'Nules' Clementine mandarin fruit were harvested one week apart but did not necessarily come from the same farm. Fruit were washed over rotating brushes using 1 $mL.L^{-1}$ didecyl dimethyl ammonium chloride (Sporekill, ICA International, Stellenbosch, South Africa), dried at ambient temperature in a drying tunnel and stored at 4°C. One day before the trial, the fruit were transferred from cold storage to 22°C.

2.2. Fungal cultures and spore suspensions

Two isolates of *P. digitatum* were used in the trials: isolate STE-U 6560 that is sensitive to TBZ and IMZ, and isolate STE-U 6590 that is resistant to TBZ and IMZ (Erasmus *et al.*, 2011). The isolates were plated out from -80°C storage cultures onto PDA (DIFCO, Becton, Dickinson and Company, USA and Le Pont de Claix, France) and grown for 7 – 14 days at 25°C before each trial. Spore suspensions were freshly prepared each day of inoculation by filtering the culture grown on PDA through two layers of cheesecloth with distilled water amended with Tween 20 (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 0.01 $mL.L^{-1}$. Spores were counted with a haemocytometer and final spore concentration adjusted to 10^6 $spores.mL^{-1}$. Viability of spores was verified after each trial by plating out the used spore suspension on PDA.

2.3. Fruit Inoculation

Fruit were inoculated by dipping a sterilized wound inducer into the spore suspension and making a 2 mm deep wound into the peel, piercing both the flavedo and albedo. Four wounds were made on each fruit equidistantly around the calyx. Wound inducers used for the dip trials consisted of three insect needles placed in a needle clamp to create three small wounds of 0.5 mm wide and 2 mm deep at a triangular distance of 1.5 mm apart. Wound inducers used for wax and drench trials consisted of a 7 mm diameter cylindrical rod with a protruding tip 2 mm long and 1 mm in diameter.

2.4. TBZ residue loading and effective residue values for curative and protective control of P. digitatum *using dip treatments*

For Clementine mandarin fruit, a 6×2×2 factorial experiment was done where fruit were dipped in one of 6 different concentrations of TBZ (0, 50, 100, 250, 500 or 1000 $\mu g.mL^{-1}$). For navel and Valencia orange fruit, a 6×2×2 factorial experiment was done where fruit were dipped in one of 6 different concentrations of TBZ (0, 50, 100, 250, 500 or 1000 $\mu g.mL^{-1}$). Trials on navel and Valencia orange fruit were repeated in a 5×2×2 factorial experiment where fruit were dipped in one of 5 different concentrations of TBZ (0, 50, 100, 1000 or 2000 $\mu g.mL^{-1}$). Fruit were inoculated with either the sensitive or resistant isolate of *P. digitatum* and treated curatively or protectively. Fruit treated curatively were inoculated 24 hours before the dip treatment. Control fruit were not dipped but only inoculated with the TBZ-sensitive (S) or TBZ-resistant (R) isolate. The inoculated fruit were placed in

cartons and covered with polyethylene bags and incubated at 22°C. Four replications were done for each treatment, with 12 fruit per treatment replication.

In order to simulate dip tanks in the packhouse, dip baths were prepared by adding 25 L municipal water into 30-L plastic containers the night before to allow the water to reach ambient temperature (22°C) overnight. TBZ (Thiazole 500 SC, Villa Crop Protection, South Africa) was added to each bath to make up concentrations of 50, 100, 250, 500, 1000 or 2000 $\mu g.mL^{-1}$. One litre of water from each bath was replaced with 1 L of hot water into which TBZ has been dissolved. One of the baths contained water only. Fruit were dipped for 60 s in the bath. Baths were stirred frequently during the trial in order to prevent any precipitation of TBZ. Six randomly selected non-inoculated fruit were sampled from each dip treatment for TBZ residue analysis. The fruit for the protective trial were allowed to dry and were then inoculated with a freshly prepared spore suspension on the same day that they were dipped. Different TBZ suspensions were used to treat fruit inoculated with the sensitive and resistant isolates. The pH of each suspension was measured after the addition of TBZ to the water with a pH meter (HI 991002, Hanna instruments, Smithfield, RI).

After treatment and inoculation the fruit were incubated at 22°C for 11 days. Infection was rated with a UV light (UV-A at 365 nm, Labino Mid-light; www.labino.com) after 4 days' incubation. The number of infected wounds was recorded using the UV light to indicate yellow fluorescent green mould lesions that could not yet be seen with the naked eye (Erasmus *et al.,* 2011). Sporulation was rated after 10 or 11 days on a scale of 0 – 6 (0 = no sign of disease; 1 = visible lesion but no sporulation; 2 = sporulating area on lesion smaller than a quarter of the fruit; 3 = sporulating area larger than a quarter of the fruit, but smaller than half of the fruit; 4 = sporulating area larger than half of the fruit, but smaller than three quarters of the fruit; 5 = sporulating area larger than three quarters of the fruit, but smaller than the whole fruit; 6 = sporulating area covering the whole fruit). Sporulation incidence was calculated for infected fruit only, with a sporulation index of 1 to 3 regarded as inhibition and 4 to 6 regarded as sporulation. The percentage sporulation incidence was used in further data analysis.

Dip trials were conducted 6 times; twice each on Clementine mandarin, navel and Valencia orange fruit.

2.5. Wax coating trials

Fruit were treated on a custom-built experimental packline (Dormas, Johannesburg, South Africa) resembling those in commercial packhouses. It consisted of four modular units: an elevator feeding fruit into the line; a re-cycling spray-on washing system over 8 brushes; a coating applicator with 8 rotating synthetic brushes and coating applicator (JBT Foodtech, Brackenfell, South Africa); and a drying tunnel that uses high volume air at low speeds (ambient temperature) to dry the fruit.

The wax coating applicator was calibrated using two pulsating nozzles (0.5 s on, 2 s off) at 22 mL.min^{-1} (3 bar). The packline is equipped with synchronised variable speed drives and the wash and coating units have brush-sweep paddles to move fruit across the unit at a set speed. As per the industry recommendation, the rotating brushes were adjusted to 100 rpm for Satsuma and Clementine mandarin fruit, and to 120 rpm for treatment of Valencia orange fruit.

Wax was prepared by adding 0.04 mL.L^{-1} anti-foaming adjuvant (Antifoam, Chempac, Paarl, South Africa) to a carnauba wax coating [Endura-Fresh 6100 (18% solids), John Bean Technologies, Brackenfell, South Africa]. Wax coating was applied on the rotating brushes for 15 s to saturate them with wax before the fruit was treated. Buffer fruit was put through the applicator before and after each replication's treatment fruit. Brushes were flushed for 5 s before increasing the wax load. Different brush sets were used for the fruit treated with wax only and fruit treated with TBZ-amended wax. Six non-inoculated fruit were added to each treatment to be used for residue analysis.

A 2×2×2×3 factorial experiment was done where fruit were treated with the non-amended carnauba wax coating or with wax coating amended with 4000 µg.mL^{-1} TBZ (ICA Thiabendazole 500 SC, ICA International, Stellenbosch, South Africa). Wax was applied at three different wax loads: 0.6 L.ton^{-1} of fruit (10 s exposure on wax applicator); 1.2 L.ton^{-1} of fruit (20 s exposure), and 1.8 L.ton^{-1} of fruit (30 s exposure). In each case the line speed was adjusted. After applying the wax coating, fruit were dried in the drying tunnel.

Curative green mould control was evaluated by treating fruit that were inoculated with freshly prepared spore suspensions of either the sensitive or the resistant isolate 24 h prior to wax coating treatment, while protective control was evaluated by inoculating fruit 1- 3 hours after waxing. Fruit were incubated and rated in the same manner as in Section 2.4.

The remainder of treated fruit (12 fruit per treatment) were not inoculated but stored at -0.5°C for 40 days to evaluate the effect of TBZ-incorporated wax treatment on chilling injury. In order to evaluate the effects of TBZ dip treatment followed by non-amended wax treatment, fruit were dipped in 1000 µg.mL^{-1} TBZ (Valencia orange fruit), or in 2000 µg.mL^{-1} TBZ (Satsuma and Clementine mandarin fruit) and were then treated with non-amended wax at a load of 1.2 L.ton^{-1} fruit. To evaluate the effects of no dip, water or TBZ dips alone, fruit were dipped either in water or in a 1000 µg.mL^{-1} aqueous TBZ suspension (Valencia orange fruit); or in 2000 µg.mL^{-1} TBZ (Clementine and Satsuma mandarin fruit), or were left untreated before cold storage. After 40 days, fruit were taken out of storage and left at 22°C for 4 days. Chilling injury was rated on a scale of 0 to 3, where 0 = no pitting; 1 = slight pitting; 2 = medium pitting, and 3 = severe pitting (Sala, 1998). Chilling inury incidence was used in further data analysis. Additionally, the effect of treatments on the preservation of fruit buttons was analysed by rating the incidence (%) of green buttons.

Four replications were done for each treatment combination, using 12 fruit per treatment combination. Controls consisted of non-waxed or waxed fruit that were inoculated 24 hours before or 1 – 3 hours after waxing of treatment fruit. Wax trials were done 6 times; twice per citrus type.

2.6. Drench trials

Unwashed fruit were packed into black plastic drench crates (480 × 310 × 280 mm; closed sides) according to replicates. Each drench crate had evenly spaced holes in the bottom to allow the TBZ suspension to run through. The holes comprised the same area compared to the holes in the bottoms of wooden commercial bins commonly used in South Africa (CHEP, Durban, South Africa). To prevent fruit blocking holes, three PVC pipes were placed at the bottom of each crate and a layer of non-inoculated buffer fruit was placed on the bottom of each drench crate. Inoculated fruit were placed in the crate with half of it facing button-up and the other button-down. Fruit were inoculated 24 h before drenching, 6 h before drenching (curative treatments) or 24 h after drenching (protective treatment), as described previously. A layer of non-inoculated fruit was placed over the inoculated fruit in the top of each crate. Fruit in each crate were drenched using an experimental drench system designed to simulate industry best-practice. The drench solution was pumped through a spray manifold into an empty drench crate at 52 $L.min^{-1}$, and showered through the evenly spaced holes in an empty crate onto the fruit in the drench crate below. The solution was collected and recirculated from a larger container in which the fruit crate was placed in an elevated position so as to prevent fruit from standing in the solution. Different exposure times (30 s, 60 s and 90 s) and different TBZ concentrations (1000 or 2000 $\mu g.mL^{-1}$) were evaluated, which simulated the industry-recommended standard of 125, 250 and 375 $L.bin^{-1}$, respectively. After drenching, fruit were left to dry overnight before being packed into boxes. Four replications were done in each trial and the trials were done twice each on Clementine mandarin and navel orange fruit.

2.7. Residue analysis

For each treatment combination, six non-inoculated fruit from two replications were sampled for residue analysis, and stored at -20°C until they were prepared for residue analysis. Fruit were chopped, then weighed and pulped for 2 minutes using a blender (Salton Elite, Almalgamated Appliance Holdings Limited, Reuven, South Africa). Pulped fruit samples were stored at -20°C. Samples from the first and last replications of each treatment were sent for TBZ residue analyses by an accredited analytical laboratory (Hearshaw and Kinnes Analytical Laboratory, Westlake, Cape Town, South Africa). Samples were extracted by using acetonitrile followed by a matrix solid phase dispersion extraction. Analysis of the extracts was conducted in liquid chromatography mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA).

2.8. Statistical analysis

For the green mould control trials (dip, drench and wax), wound infection data were normalized by calculating the percentage control relative to the untreated control. For the chilling injury trial, the percentage incidence data were used. Data were subjected to analysis of variance, means were separated using Fisher's least significant difference test at 95% confidence interval, and trends were described using appropriate regression analyses in XLSTAT version 2011.4.02 (www.xlstat.com).

3. Results

3.1. TBZ residue loading and effective residue levels for curative and protective control of P. digitatum *using dip treatments*

Analysis of variance (ANOVA) for residue data was done separately for each citrus type. For Clementine mandarin fruit, there was a significant interaction between trial and TBZ concentration ($P < 0.0001$, Annex A, Table 1). Fisher's LSD showed that there were no differences in terms of residue loading between the two trials at all TBZ concentrations used, except at the lowest and highest concentrations (50 and 1000 $\mu g.mL^{-1}$ TBZ). These differences were less than 0.15 $\mu g.g^{-1}$ (results not shown), and trends were similar, thus the trial factor was ignored in further analysis of the data. For navel oranges, an interaction between trials and TBZ concentration was not observed ($P = 0.9456$), and only TBZ concentration had an effect ($P < 0.0001$). For Valencia orange fruit, there was also an interaction between trial and TBZ concentration ($P = 0.0137$), and significant differences occurred between trials at all the TBZ concentrations used. Higher residues were loaded in the second trial, but to describe a more robust residue loading trend, data were combined. TBZ residue loading on Clementine mandarin, navel and Valencia orange fruit followed linear trends (R^2 values = 0.77, 0.94, 0.76, respectively; Fig. 1). Clementine mandarin fruit loaded TBZ residue levels at a slower rate and had a predicted level of 0.5 $\mu g.g^{-1}$ following dips at 1000 $\mu g.mL^{-1}$ for 60 s. Navel and Valencia orange fruit showed markedly faster loading rates, with residue levels predicted following a 60 s dip in 1000 $\mu g.mL^{-1}$ TBZ of 1.5 and 1.4 $\mu g.g^{-1}$, respectively. All aqueous TBZ suspensions had a pH of 6.99 – 7.00.

Analysis of variance of percentage control data showed a significant interaction between trial, treatment (TBZ concentration), action and isolate for all citrus types ($P = 0.0015$, $P = 0.0036$ and $P < 0.0001$ for Clementine mandarin, navel and Valencia orange fruit, respectively; Annexure A, Table 2). Differences between trials were largely ascribed to susceptibility differences between fruit batches.

Non-linear regression was done with curative and protective control of the sensitive and resistant isolate as response variables and with TBZ residue level as independent variable. For Clementine, navel and Valencia, very good fits were observed for curative control data of the sensitive isolate using an exponential association model, y=a*(1-exp(-bx)); (R^2 = 0.70, 0.86 and 0.97,

respectively; Table 1). Effective TBZ residue levels indicating 50% and 75% curative or protective green mould were calculated from the type-specific model. Low residue levels of TBZ were needed to obtain 50% and 75% curative control of the sensitive isolate (0.11, 0.07, 0.03 µg.g^{-1} and 0.22, 0.13, 0.06 µg.g^{-1} for Clementine mandarin, navel and Valencia orange fruit, respectively).

Accurate models could not be fitted to protective treatment data, and also not for control of the resistant isolate (R^2 = 0.01 – 0.53 for the exponential association model; results not shown). For navel orange fruit, minor differences were observed between trials, and protective treatments with 1000 and 2000 µg.mL^{-1} TBZ led to 36.6 and 55.1% control for the sensitive isolate, compared to 99.5 and 98.4% for curative treatment. Curative and protective control of the resistant isolate ranged from 0 – 18.7%. For Valencia orange fruit, significant differences were observed between trials. The protective treatments in the first trial showed significantly better control than in the second trial (62.5 – 97.9% *vs*. 6.2 – 50.1%). Interestingly, TBZ residue levels on treated Valencia fruit were significantly higher in the second trial than the first trial (0.15 – 2.10 µg.g^{-1} *vs.* 0.08 – 0.85 µg.g^{-1} following TBZ dip treatments in 50 to 1000 µg.mL^{-1}). These marked differences in control between trials were not observed for the curative treatments (81.7 – 100% and 90 – 97.3% control, respectively). For the resistant isolate, better control was also observed following protective (31.9 – 55.2% *vs.* 0 – 4.2%) and curative (19.1 – 49.4% *vs.* 0 – 4.6%) treatments in the first trial compared with the second trial respectively. For Clementine mandarin fruit, trial interactions were ascribed to minor differences between individual treatments that were observed, and not to marked differences between trials; the trial effect was therefore ignored. At 1000 µg.mL^{-1} TBZ, protective treatments of the sensitive isolate led to 17.1% control compared to 91.3% control of the curative treatments. The resistant isolate was poorly controlled on Clementine mandarin fruit with no significant difference between curative (1.9 – 13.3%) and protective (12.3 – 17.4%) treatments.

For sporulation incidence, analysis of variance showed a significant interaction between treatment, action and isolate for navel and Valencia orange fruit (P < 0.0001 and P = 0.0022, respectively; Annexure A, Table 3). Generally, sporulation incidence decreased with increasing TBZ concentration. For Clementine mandarin fruit, a significant interaction was observed between trial, treatment and isolate (P = 0.0096). Lower sporulation incidence was seen in the second trial compared to the first trial. For navel orange fruit, sporulation incidence was only reduced at 1000 and 2000 µg.mL^{-1} TBZ, for both curative and protective control of the sensitive isolate (Table 2). At 2000 µg.mL^{-1} TBZ, the best results were seen for sporulation incidence (21.2 and 57.4% incidence for curative and protective treatments respectively, compared to 99.5 – 100% on untreated control fruit). On Valencia orange fruit, data were variable, but the best results were seen at 2000 µg.mL^{-1} TBZ for curative and protective control of the sensitive isolate (60.2% and 29.4%, respectively, compared to 92.5 – 99.5% on control fruit). On Clementine mandarin fruit, significant lower

sporulation incidences were seen at 1000 µg.mL^{-1} TBZ for curative and protective treatment of the sensitive isolate (95.5% for curative and 95.7% for protective treatments at 1000 µg.mL^{-1} TBZ, compared to 99.5 – 100% on control fruit).

3.2. Wax coating trials

Analysis of variance of TBZ residue level data showed a significant interaction (P = 0.0009; Annexure A, Table 4) between citrus type and treatment. Valencia orange fruit loaded significantly higher residue levels than Satsuma and Clementine mandarin fruit following TBZ amended wax treatment (Table 3) and frequently exceeded the MRL of 5 µg.g^{-1} at wax loads of 1.2 and 1.8 L.ton^{-1}. Residue loading on the two soft citrus types did not differ significantly. TBZ residue levels increased with increasing wax load: from 0.40 to 3.10 µg.g^{-1} on Clementine mandarin fruit, 0.40 – 2.90 µg.g^{-1} on Satsuma mandarin fruit, and 2.70 – 7.60 µg.g^{-1} on Valencia orange fruit. TBZ residue loading following increasing wax loads followed linear trends (R^2 = 0.66, 0.60 and 0.80 for Clementine, Satsuma mandarin and Valencia orange fruit, respectively; results not shown). For Satsuma mandarin fruit, the rate of residue loading followed the equation y = 1.3893x, for Clementine mandarin fruit y = 1.4591x and for Valencia orange fruit y = 4.4032x (where y is the residue loaded in ug.g^{-1} and x is the wax load in L.ton^{-1}). Valencia orange fruit had a much faster rate of residue loading than the soft citrus types.

Given the variable infection data obtained on untreated control fruit, percentage infection data was used rather than percentage control data. Analysis of variance showed a significant interaction between citrus type, treatment (wax load combined with TBZ concentration), action (curative or protective) and isolate (sensitive or resistant isolate of *P. digitatum*) (P < 0.0001; Annexure A, Table 5). High infection levels were observed with the resistant isolate and no significant differences were generally observed between control, non-amended and TBZ amended wax treatments on all citrus types (Table 4). For the sensitive isolate, better protective than curative control was generally observed with TBZ applied in wax. On Valencia orange fruit, protective treatment with TBZ led to 14.1 – 19.5% infection compared with 89.6% on control fruit and 91.1 – 94.8% on waxed control fruit; no significant difference was observed between TBZ amended wax coating loads. Curative treatment with TBZ in wax resulted in somewhat higher infection levels, with significantly better control at 1.2 and 1.8 L wax.ton^{-1} fruit compared with 0.6 L wax.ton^{-1} fruit (26.8 – 27.1% *vs.* 40.4% respectively). For the two soft citrus types, infection levels were only slightly reduced by TBZ amended wax treatments. Satsuma mandarin fruit showed the highest infection of all citrus types, with protective TBZ wax treatments ranging from 87.0 – 90.1% infection compared with 91.1% on control fruit and 92.2 – 96.6% on waxed control fruit. Curative TBZ in wax treatment resulted in 92.2 – 95.3 % infection. There was no significant difference between wax coating loads or between treated and control fruit. On Clementine mandarin fruit, protective TBZ wax treatments led

to 58.9 – 87.8% infection compared with 90.9% on control fruit and 83.3 – 91.1% on waxed control fruit. Curative TBZ in wax treatment resulted in 81.5 – 90.4% infected fruit and infection decreased significantly at the highest wax load.

For sporulation incidence, analysis of variance showed a significant interaction between citrus type, treatment (wax load combined with TBZ concentration), action (curative or protective) and isolate (sensitive or resistant isolate of *P. digitatum*) ($P < 0.0001$; Annexure A, Table 5). Sporulation incidence was near 100% for all infected non-amended waxed or untreated control fruit. For the resistant isolate, close to 100% sporulation incidence was observed in all treatments. For the sensitive isolate, significantly lower incidence of sporulation was observed when Clementine or Satsuma mandarin fruit were treated curatively (0.0 – 41.7% and 0.0 – 22.8%, respectively; Table 5) compared to protective treatment (12.6 – 73.3% and 8.7 – 54.8%) at 1.2 and 1.8 L wax.ton^{-1} loads. To the contrary, sporulation incidence of the sensitive isolate on Valencia orange fruit was generally lower on protectively (3.4 - 43.6%) than curatively treated fruit (9.2 - 61.1%) across all wax loads. Treatment with TBZ amended wax generally resulted in a decreasing sporulation incidence with increasing wax load.

Analysis of variance showed that the trial × treatment interaction was significant ($P < 0.0001$; ANOVA not shown) for chilling injury (CI) incidence. Trials with the same citrus type were compared and differences were observed between the two Valencia trials, where treatments in the second trial led to much lower CI incidence than in the first trial. Control fruit in the Satsuma trials had different levels of CI and therefore all the treatments differed between the two trials. The two Clementine trials showed similar results. Since trends were the same, this interaction was ignored, and data for each citrus type were grouped and analysed together. Analysis of variance showed a significant interaction between citrus type and treatment ($P < 0.0001$; Annexure A, Table 6). Satsuma and Clementine mandarin fruit showed a low incidence of chilling injury on control fruit, and none of the treatments lowered this incidence significantly. Control fruit from the Valencia trials showed a high amount of chilling injury (79.2%; Table 6), and this was significantly lowered by all the treatments, with the exception of dipping fruit in cool water. Non-amended wax treatments at 0.6 and 1.2 L.ton^{-1} and TBZ dip treatment led to a significantly higher incidence of CI than the other TBZ-amended wax coating treatments (44.8 – 47.2% *vs.* 18.8 - 27.1%). TBZ wax at 1.8 L.ton^{-1} and the TBZ dip followed by non-amended wax treatments at 1.2 L.ton^{-1} showed the lowest mean CI incidence (18.8 and 18.1%, respectively).

Analysis of variance showed a significant interaction between citrus type and treatment for amount of buttons that remained green during storage ($P < 0.0001$; Annexure A, Table 7). On control fruit, Valencia orange fruit had the highest incidence of green buttons (83.3%; Table 7), followed by

Clementine mandarin fruit (77.8%). Satsuma mandarin fruit had a very low incidence (8.3%). Non-amended wax had a beneficial effect on button appearance of soft citrus: 36.5 – 37.5% for Satsuma mandarin fruit at 0.6 – 1.8 L.ton^{-1} and 96.9% for Clementine mandarin fruit at 1.2 and 1.8 L.ton^{-1}, but significantly lower levels of green buttons at 0.6 L.ton^{-1} (76.7%). On Valencia orange fruit, dip treatments seemed to have a detrimental effect (47.2%). TBZ-amended wax led to significantly lower green button incidence than non-amended wax treatments on Satsuma mandarin fruit, while on Clementine mandarin fruit and Valencia orange fruit the results were similar.

3.3. Drench trials

Navel fruit. Analysis of variance for TBZ residue values on navel orange fruit showed no interaction between TBZ concentration and exposure time (P = 0.7257, Annexure A, Table 8); TBZ concentration had a significant effect on residue loaded (P = 0.0023), but the effect of exposure time was not significant (P = 0.5498). A TBZ concentration of 2000 µg.mL^{-1} loaded significantly higher residues than 1000 µg.mL^{-1} TBZ, (mean of 5.40 *vs.* 3.50 µg.g^{-1}) and often exceeded the MRL of 5 µg.g^{-1}.

Analysis of variance of control data from navel orange fruit showed a significant interaction between action and orientation (P < 0.0001; Table 9), and between TBZ concentration and action (P = 0.0035). Disease was significantly better controlled on fruit with wound sites facing to the top (button-up) than fruit facing down, for 24 h curative (89.7 *vs.* 67.7%, respectively), 6 h curative (92.1 *vs.* 77.2%) as well as for protective treatments (37.9 *vs.* 31.8%). For the curative treatments, there was no significant difference in the percentage control obtained, whether 1000 or 2000 µg.mL^{-1} TBZ was used (77.7 - 79.6% and 83.9 – 85.4% for 24 h and 6 h curative treatments, respectively). However, for the protective treatments, 2000 µg.mL^{-1} TBZ resulted in a higher percentage control (39.8 *vs.* 29.9%). Exposure time had a significant effect (P = 0.0002): drenching for 30 s led to significantly poorer control (62.0%) than drenching for 60 or 90 s (67.2 - 69.0%). Poor correlations were obtained by non-linear regression analysis (exponential association model) for residue loaded and percentage control obtained (R^2 ranging from 0.05 – 0.11).

Analysis of variance of sporulation incidence data from navel orange fruit indicated interactions between exposure time and action (P = 0.0100; Table 10) and an interaction between TBZ concentration and exposure time (P = 0.0366). At 1000 µg.mL^{-1}, 90 s exposure time led to a significantly higher sporulation incidence than 60 s or 30 s (53.5 *vs.* 37.2 – 38.9%, respectively). At 2000 µg.mL^{-1} there were significant differences between all the exposure times (20.1, 32.6 and 43.1% for 30, 60 and 90 s, respectively). There was no significant difference between exposure times for 24 h curative treatments (27.1 – 39.6%), but for 6 h curative treatments, 30s exposure time led to significantly less sporulation incidence than 60 and 90 s (28.6 *vs.* 42.2 – 50.5%, respectively). For

protective treatments, drenching for 90 s led to significantly higher sporulation (54.7%) than drenching for 30 or 60 s (28.6 – 32.8%).

Clementine fruit. On Clementine mandarin fruit, exposure time did not have a significant effect (P = 0.1933; Table 11) with residues increasing only marginally from 30 to 90 s exposure at 1000 µg.g^{-1} (2.02 to 2.25 µg.g^{-1}). TBZ concentration appeared to have some effect as 45 s exposure to 2000 µg.mL^{-1} resulted in higher residues (3.06 µg.g^{-1}) than exposure for 30, 60 or 90 s at 1000 µg.mL^{-1} (2.02 – 2.25 µg.g^{-1}).

Analysis of variance of control data showed a significant interaction between orientation and action (P = 0.0183; Table 12), and between treatment and action (P = 0.0010). Fruit facing button-up led to better disease control than fruit facing button-down for 24-h curative (55.8 *vs.* 33.9%), 6-h curative (84.4 *vs.* 66.3%) and protective treatments (17.1 *vs.* 9.3%). There was no significant difference between treatments (consisting of fruit drenched with 1000 µg.mL^{-1} TBZ for 30 s, 1000 µg.mL^{-1} TBZ for 60 s, 1000 µg.mL^{-1} TBZ for 90 s, or 2000 µg.mL^{-1} TBZ for 45 s) for the 6-h curative action (71.6 – 77.6% control). For curative 24 h action the only treatments that differed were1000 µg.mL^{-1} for 30 s and 2000 µg.mL^{-1} for 45 s (36.2 *vs.* 54.9%) Relatively poor protective control was achieved with the 1000 µg.mL^{-1} for 30 s treatment giving the highest control (21.6%) of all the treatments, and differed from all the other protective treatments (6.0 – 12.9%).

Analysis of variance showed a significant interaction between treatment and action (P = 0.0003; Table 13) for sporulation incidence. For 24 h curative treatments, similar sporulation incidence was observed for the 1000 µg.mL^{-1} drench for 30 s and 2000 µg.mL^{-1} drench for 45 s (39.4 – 48.2%), and this differed significantly from the 1000 µg.mL^{-1} drenches for 60 s and 90 s (65.8 – 70.0%). For 6 h curative treatments, sporulation incidence was less after the 2000 µg.mL^{-1} drench for 45 s (35.1%) than the 30, 60 and 90 s treatments at 1000 µg.mL^{-1} (53.8 – 57.4%). Protective treatments showed markedly lower sporulation incidence at the 2000 µg.mL^{-1} drench for 45 s (25.0%) compared with the 30, 60 and 90 s treatments at 1000 µg.mL^{-1} (72.6 – 90.4%).

4. Discussion

This study clearly indicated differences in TBZ residue loading and green mould control between different methods of application commonly used in South Africa. The study also showed that the TBZ resistant isolate could not be controlled with TBZ.

TBZ applied as a dip treatment loaded the lowest residues compared with drench and wax application, but it gave the best curative control of green mould, and relatively poor or unreliable protective control on all citrus types. When TBZ was applied in a wax coating, protective treatments

led to better control than curative treatments, although control was relatively poor. The drench application of TBZ was most effective if fruit were drenched 6 h after inoculation, and its efficacy diminished when drenched 24 h after inoculation, especially on Clementine mandarin fruit. Protective drench treatments were not effective. Protective control seemed especially dependent on the fruit batch, but TBZ did not provide a good, reliable protective action in any of the applications. TBZ was also shown to reduce chilling injury incidence in wax coating application.

For all three citrus types, the residue levels loaded when dipped in 1000 $\mu g.mL^{-1}$ TBZ for 60 s at 22°C and pH 6.99 were well below the MRL of 5 $\mu g.g^{-1}$, and ranged from 0.50 – 1.60 $\mu g.g^{-1}$. At the recommended concentration of 2000 $\mu g.mL^{-1}$ TBZ the MRL was not exceeded and ranged from 2.90 – 3.10 $\mu g.g^{-1}$ on Valencia and navel orange fruit. Schirra found 1.59 $\mu g.g^{-1}$ following dips for 60 s in 600 $\mu g.mL^{-1}$ at 20°C, and pH of 7.04. A linear relationship was found between TBZ concentration and the amount of TBZ residue loaded on fruit in this study. Smilanick *et al.* (2006b) also found a linear relationship between TBZ concentration and residues loaded on lemons, but found a faster rate of loading compared to our study on Clementine mandarin, navel and Valencia orange fruit (slope of 0.004 *vs*. 0.0005 to 0.0015). The difference in TBZ residue loading rates could be attributed to fruit type, as was also evident in the 3-fold faster loading rate observed on orange fruit compared with Clementine mandarin fruit in our study. Schirra *et al.* (2008) loaded 1.1 – 1.5 $\mu g.g^{-1}$ TBZ residues at 600 $\mu g.mL^{-1}$ at 20°C on Valencia orange fruit, rather than a predicted 0.84 $\mu g.g^{-1}$ TBZ in our study. However, they reported that Valencia orange fruit loaded more residues than Salustiana orange fruit, which again demonstrates the influence of citrus type on residue loading. Residue loading with pyrimethanil was also found to be influenced by citrus type (Smilanick *et al.,* 2006). Imazalil residue loading was not influenced by citrus type according to Erasmus *et al.* (2011), except when imazalil sulphate was applied at an increased pH (Erasmus *et al.,* 2013). Smilanick *et al.* (1997) found that different citrus types did indeed load different IMZ residues.

Nordby and Nagy (1977) showed that the epicuticular wax of different citrus cultivars differed significantly with regards to the type of hydrocarbons found in them. A change in epicuticular wax of citrus fruit was observed by Palma *et al.* (2013) after treatment with TBZ. The use of adjuvants alters the epicuticular wax by solubilizing certain components (Tamura *et al.,* 2001), and it was suggested that the change in epicuticular wax appearance is due to the adjuvants in the TBZ formulation, and not necessarily due to TBZ itself (Palma *et al.,* 2013). This might explain why higher residues were loaded on fruit in other studies; for example, Schirra *et al.* (2008), D'Aquino *et al.* (2013), Hordijk *et al.* (2013) and Palma *et al.* (2013) all used Tecto® (thiabendazole, Syngenta) in their studies and loaded higher TBZ residues than found in our study, which used Thiazole® (thiabendazole, Villa Crop Protection, South Africa).

The location of TBZ deposition on the fruit skin was found to be mostly on the outer surface (Schirra *et al.,* 2008), which contributed to the hypothesis that the condition and composition of the epicuticular wax influence residue loading. Schirra *et al.* (2008) also found that the location of the TBZ residue on the fruit is very important, *i.e.* whether it is superficially on the rind, absorbed into the cuticle or even deeper into fruit tissue. Since TBZ has a tendency to precipitate, it is important to keep the suspension well agitated, as was done in the studies of Schirra *et al.* (2008) and D'Aquino *et al.* (2013). It should be noted that no excessive precipitation of TBZ was observed during the experimentation in this study.

Schirra *et al.* (1998) also found that the fruit rind condition at harvest influenced TBZ residue loading, which might explain differences between fruit batches of the same type. In our study, different fruit types were treated at different times, and it would be interesting to simultaneously investigate residue loading on different fruit types of similar maturity standards. It is known that the half-life of pesticides differ among plant species (Fantke and Juraske, 2013). Since samples were not processed immediately, this might have played a role in this study.

When applied as a dip treatment, TBZ performed best when used curatively. This confirmed the work of Smilanick *et al.* (2006). Effective residue levels for 50% control ranged from 0.03 – 0.11 $\mu g.g^{-1}$ and were 0.06 – 0.22 $\mu g.g^{-1}$ for 75% control depending on citrus type. This is much lower than effective residue levels for IMZ; a predicted 0.23 $\mu g.g^{-1}$ IMZ was needed to obtain 50% control (Erasmus *et al.,* 2013). Gutter (1970) found that infection in curatively treated Valencia orange fruit only lowered from 8.1 to 4.6% when TBZ concentration was increased from 500 to 2000 $\mu g.mL^{-1}$. This corresponds with the results of this study that showed a maximum level of control is already obtained at very low residues. Even though navel orange fruit loaded the highest residue, Valencia orange fruit generally showed better control (and therewith lower effective residue values for curative control). Protective control was generally poor; however, on Valencia fruit this was dependent on fruit batch. Valencia fruit in the second trial loaded markedly higher residues, but the protective control was considerably lower than in the first trial in which acceptable protective control levels were obtained. Although this was not measured, the differences between trials were most probably linked to fruit quality differences, which highlights the importance of fruit quality on the ability to control green mould. Other trials have also indicated that poor quality fruit loaded higher residues than better quality fruit, but higher residues on poor quality fruit did not lead to improved control (P.H. Fourie, *unpublished results*). The fruit rind condition at harvest is known to influence TBZ residue loading (Schirra *et al.,* 1998), but the link between fruit quality, residue loading and green mould control needs to be investigated further. Schirra *et al.* (2008) and Erasmus *et al.* (2011) also reported that Valencia orange fruit were naturally less susceptible to green mould than other citrus types. It must be kept in mind that different pre-harvest practices may occur for different fruit

cultivars, such as choice of rootstock, irrigation, fertilization and harvesting practices, which could influence the fruit's susceptibility to green mould; it can also explain the variability in results between different batches of fruit (McDonald and Wutscher, 1974; Michailides *et al.,* 2010).

Our methodology evaluated TBZ protection of intact citrus fruit rinds against green mould infection from freshly made wounds. In this case, TBZ in the dip did not perform well as a protective treatment; moreso on Clementine and navel fruit than on Valencia orange fruit. The reason for the low protective action is probably due to the limited penetration of TBZ into the fruit rind, meaning that any new wounds would be unprotected. It is anticipated that TBZ protection of non-infected fresh rind wounds against subsequent infection would be very effective, as was seen for the curative treatments. However, this hypothesis should still be tested. Electron micrographs showed that the epicuticular wax on citrus fruit becomes 'patchy' after TBZ treatment (D'Aquino *et al.,* 2013; Palma *et al.,* 2013). Whether the uneven distribution of epicuticular wax influences the uniformity of TBZ deposition is not known; it is anticipated that improved deposition uniformity should lead to improved control (van Zyl *et al.*, 2010). Contrary to our findings, El-Tobshy *et al.* (1987) found that dipping fruit in aqueous TBZ did give good protective control, but curative treatments were still more effective. It should be noted that they used dry spores for inoculum and the disease pressure was probably lower than in this study.

Imazalil also gave better curative than protective control in the dip application (Dore *et al.,* 2009; Erasmus *et al.,* 2011), but protective treatments with imazalil were more effective than the protective treatments with TBZ in this study. Pyrimethanil was also found to have an excellent curative action (Smilanick *et al.,* 2006), and a less effective protective action (P.H. Fourie, *unpublished results*). When applied as a curative treatment, pyrimethanil and imazalil reduced decay completely, while azoxystrobin and fludioxonil only reduced decay up to approximately 40% and 20%, respectively (Kanetis *et al.,* 2007). On naturally infected fruit, the curative action of TBZ was similar to that of imazalil and fludioxonil (Zhang, 2007). Guazatine was more effective than TBZ as a curative dip treatment (Kassim and Khan, 1996), but Wild and Spohr (1989) found that its efficacy was less than TBZ when incubation times were prolonged. Sodium ortho-phenylphenate (SOPP) reduced decay up to 20% and less sporulation incidence was observed when fruit were treated curatively (Hall, 1978). TBZ was more effective in reducing natural decay compared to SOPP (Smoot and Melvin, 1970). Sporulation incidence following TBZ dip treatments was generally very high as seen on Clementine mandarin fruit, as well as for the protective dip treatments of navel and Valencia orange fruit.

When TBZ was incorporated in wax coating application, it led to much higher residues on all citrus types. TBZ applied in wax at 4000 $\mu g.mL^{-1}$ resulted in exceedence of the MRL when applied at

higher wax loads (1.2 and 1.8 L wax.ton^{-1} fruit) on Valencia orange fruit. The observation that Valencia orange fruit loaded higher residues than the soft citrus may be due to various factors, including the size of the fruit, and also the faster brush speed used, and needs to be investigated further.

TBZ in wax coating gave better protective than curative control of the sensitive isolate. The opposite was found by Brown (1984), i.e. better curative control in the wax, but different inoculation techniques were used. At a lower spore concentration (10^4 spores/mL), Kouassi *et al.* (2012) found that TBZ in wax at a 0.4% concentration gave 100% protective control. Unfortunately they did not specify which citrus type was used and only referred to fruit as oranges. The observation that TBZ in wax applications did not give such excellent control as in the dip, even though much higher TBZ residues were loaded was also found by Brown (1984) and El-Tobshy *et al.* (1987) who suggested that TBZ becomes encapsulated in the wax and limits its bioavailability. Non-amended wax did not have any beneficial effect on green mould control. Ansari and Feridoon (2008) also found that non-amended wax did not control decay.

In our study, very poor green mould control was observed on Satsuma mandarin fruit. Njombolwana *et al.* (2013a) also found that IMZ applied in a wax coating on soft citrus, especially Satsuma mandarin fruit, showed lower control than other citrus types. Valencia orange fruit loaded TBZ residues that exceeded the MRL at higher wax loads, and curative control increased with the increased TBZ residue resulting from increasing wax load. Njombolwana *et al.* (2013b) observed through SEM imaging that the wax coating on Valencia orange fruit was not evenly distributed at 0.6 L.ton^{-1}. Applying wax at this load may therefore lead to unreliable control.

Hall *et al.* (1978) found that protective treatment of fruit with 5000 µg.mL^{-1} TBZ in a wax treatment inhibited sporulation of TBZ-sensitive isolates to some extent. In our study, sporulation incidence was lower on soft citrus fruit following curative compared to protective treatments, and tended to be lower at higher wax loads and residue levels. The opposite was seen on Valencia orange fruit: lower sporulation incidence was observed on protectively treated fruit compared to curatively treated fruit.

Compared with TBZ in dip and wax application, drench treatments were not very effective. Higher residues were loaded in drench treatments compared with dip application, and on navel orange fruit the MRL was sometimes exceeded. However, lower levels of curative control were achieved following drench treatments than in the dip application, as well as lower levels of protective control than in the wax coating application. Increased residues led to increased protective control, but not increased curative control. Erasmus *et al.* (2011) also found that applying imazalil in the drench was not as effective as applying it in the dip tank, even though higher residue levels were

loaded on different citrus types compared to the dip applications. However, it can be expected that deposition of the residue on the fruit rind is not as evenly distributed as would be expected from dip treatments. The finding that fruit facing button-up, *i.e.* wound sites facing upward, had less disease incidence is evidence of this statement. Highly variable coverage of fungicide on fruit following drench treatment was observed using fluorescent pigment as tracer in the drench mixture (P.H. Fourie, unpublished results), which might explain why no correlation could be drawn between residue loading and green mould control following drench treatment. Interestingly, sporulation incidence seemed to decrease with decreasing drench exposure time. However, sporulation incidence was not negligible, making reliance solely on TBZ for sporulation control not feasible.

Thiabendazole inhibited chilling injury on Valencia orange fruit and benefits were similar irrespective of whether fruit were dipped in TBZ and then waxed, or whether TBZ was applied in the wax. Schiffman-Nadel *et al.* (1975) found that incorporating TBZ in wax was more beneficial for chilling injury inhibition than dipping grapefruit in TBZ and then waxing. Application of TBZ in warm water (45°C) compared to cold water (10°C) significantly reduced the incidence of chilling injury of navel orange fruit (Hordijk *et al.,* 2013). In our study, wax had an additive effect in reducing chilling injury compared to TBZ dip treatment alone, most likely due to inhibition of moisture loss and prevention of transpiration (Wang, 1993 and Wild, 1993). Likewise, wax-only treatments also inhibited chilling injury, although at lower levels than when TBZ was included. Schirra *et al.* (2000) also found that dipping grapefruit in TBZ reduced chilling injury, while Dou (2004) found that carnauba wax reduced chilling injury on its own. The addition of wax seemed to have a preserving effect on fruit buttons, but the condition of fruit at harvest probably plays a greater role, as can be concluded from the poor results on Satsuma mandarin fruit.

The TBZ resistant isolate was very poorly controlled in all applications. Extra measures are needed to control resistant isolates, such as modification of pH and temperature of the dip tank (Schirra *et al.*, 2008). Little work has been done on modification of wax coating application parameters in order to control resistant isolates. Nelson (1984) found that adding potassium sorbate to TBZ amended wax controlled resistant isolates of *P. digitatum,* but dipping fruit in an aqueous suspension of potassium sorbate followed by TBZ amended wax had the lowest amount of infection. High sporulation incidence was seen for the resistant isolate in the dip application. For wax coating application, sporulation actually increased when TBZ was applied at increasing wax loads.

TBZ is classified as a high risk for development of fungicide resistance (Brent and Hollomon, 1998). TBZ resistance in *P. digitatum* populations has been isolated with varying frequencies around the world (Holmes and Eckert, 1999; Kinay *et al*., 2007; Lee *et al.*, 2011; Sánchez-Torres and Tuset, 2011). The current status of resistance in South Africa is unknown. Due to a zero tolerance to citrus

black spot (CBS) in the European Union (EU), related benzimidazole compounds, such as benomyl and carbendazim, may be applied in citrus orchards to control CBS (Kellerman and Kotze, 1979). Smilanick *et al.* (2006) warned that frequent preharvest application of benzimidazole fungicides may lead to the increase of resistant *P. digitatum* isolates in orchards, to which postharvest application of TBZ in the packhouse will be ineffective.

The question remains as to which application of TBZ would be most beneficial in a packhouse. TBZ in the dip tank showed the highest efficacy for both green mould control (curatively) and chilling injury (if followed by a non-amended wax treatment). However, TBZ is not a popular ingredient in dip tank mixtures. In fungicide dip tanks of neutral pH and in water-based wax, TBZ tends to precipitate (McCornack, 1970; Ladaniya, 2008). In South Africa, TBZ is often added to pre-packhouse drench mixtures as it is also registered for controlling latent pathogens, such as *Colletotrichum gleosporioides* and *Diplodia natalensis*. However, since TBZ does not optimally control green mould in the drench, there will be a high risk of TBZ resistance development in *P. digitatum* populations, if TBZ-drenched fruit were submitted to the conditions of degreening, which are very favourable for green mould development. Therefore, if TBZ is used in the drench to control green mould and latent pathogens, further reliance on TBZ after degreening to control green mould should be discouraged. Moreover, use of another green mould fungicide in combination with TBZ should be advised in the drench to improve green mould control and to prevent resistance development.

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Table 1.

Non-linear regression using an exponential association model indicating the effect of thiabendazole (TBZ) residues on curative control of green mould following inoculation of Clementine, navel and Valencia fruit with a sensitive isolate of *Penicillium digitatum* and dip treatments at different TBZ concentrations (0 to 2000 µg.mL^{-1}) and incubation at 22°C for 4-5 days. Effective residue levels for 50% and 75% curative control were calculated from the equations.

			Effective residue levels (µg.g^{-1})	
Citrus type	**Equation of the model**	**R^2 value**	**TBZ-C_{50} [b]**	**TBZ-C_{75} [c]**
Clementine	C[a] = 99.66*(1-Exp(-6.39*R[d]))	0.70	0.11	0.22
Navel	C = 102.18*(1-Exp(-10.24*R))	0.86	0.07	0.13
Valencia	C = 96.82*(1-Exp(-23.54*R))	0.97	0.03	0.06

[a]C = percentage of control

[b]C_{50} = TBZ residue needed to obtain a predicted 50% curative control

[c]C_{75} = TBZ residue needed to obtain a predicted 75% curative control

[d]R= Residue loaded on fruit (µg.g^{-1})

Table 2.

Mean sporulation incidence (%) following curative and protective dip treatments of Clementine mandarins, navel and Valencia oranges at different thiabendazole (TBZ) concentrations (0 to 2000 µg.mL^{-1}) inoculated with either a sensitive or resistant isolate of *P. digitatum*. Fruit were incubated at 22°C for 10 - 11 days before sporulation was recorded and incidence (%) calculated.

	Sporulation incidence (%)[a]					
	Clementine		**Navel**		**Valencia**	
	Sensitive isolate	**Resistant isolate**	**Sensitive isolate**	**Resistant isolate**	**Sensitive isolate**	**Resistant isolate**
Curative control						
0 µg.mL^{-1} TBZ	100.0a	95.3d	99.5a	100.0a	99.5ab	91.8b
50 µg.mL^{-1} TBZ	100.0ab	99.0abc	92.9a	98.9a	94.4b	97.8ab
100 µg.mL^{-1} TBZ	97.7abcd	100.0ab	99.9a	100.0a	95.3b	97.8ab
250 µg.mL^{-1} TBZ	100.0ab	100.0ab	100.0a	98.9a	98.1ab	78.5c
500 µg.mL^{-1} TBZ	97.8abcd	97.9abcd	100.0a	100.0a	64.8cd	100.0a
1000 µg.mL^{-1} TBZ	95.5d	100.0ab	71.2cd	96.9a	98.1ab	90.2b
2000 µg.mL^{-1} TBZ	-	-	21.2e	96.9a	60.2d	90.2bc
Protective control						
0 µg.mL^{-1} TBZ	99.5abc	97.4bcd	100.0a	91.9a	92.5b	97.3b
50 µg.mL^{-1} TBZ	100.0ab	97.9abcd	100.0a	90.6ab	96.9b	98.9ab
100 µg.mL^{-1} TBZ	100.0ab	100.0ab	95.8a	83.0bc	97.2b	96.6b
250 µg.mL^{-1} TBZ	100.0abc	100.0ab	100.0a	45.7de	100.0ab	93.6b
500 µg.mL^{-1} TBZ	96.8cd	99.0abc	100.0a	87.6abc	97.6ab	97.9ab
1000 µg.mL^{-1} TBZ	95.7d	100.0ab	81.9c	96.8a	81.2c	97.9ab
2000 µg.mL^{-1} TBZ	-	-	57.4d	96.8a	29.4d	64.5d

[a]For each fruit type, means followed by the same letter do not differ significantly.

Table 3.

Mean thiabendazole (TBZ) residue levels following dip and wax coating applications on Clementine, Satsuma and Valencia fruit with treatments consisting of three different wax loads (0.6, 1.2 or 1.8 L.ton^{-1}) incorporated with a TBZ concentration of 0 or 4000 µg.mL^{-1}, or dipping fruit into an aqueous TBZ suspension of 1000 µg.mL^{-1} (Valencia oranges) or 2000 µg.mL^{-1} (Satsuma and Clementine mandarins) followed by non-amended wax coating treatment of 1.2 L/ton.

	TBZ residues (µg.g^{-1})[a]		
Treatment	**Clementine**	**Satsuma**	**Valencia**
Wax : 0 µg.mL^{-1} TBZ at 1.2 L.ton^{-1}	0.0f	0.0f	0.0f
Wax : 4000 µg.mL^{-1} TBZ at 0.6 L.ton^{-1}	0.4f	0.4f	2.7cd
Wax : 4000 µg.mL^{-1} TBZ at 1.2 L.ton^{-1}	1.3ef	1.3ef	5.7b
Wax : 4000 µg.mL^{-1} TBZ at 1.8 L.ton^{-1}	3.1c	2.9cd	7.6a
Dip : 1000 µg.mL^{-1} TBZ	-	-	0.9ef
Dip : 2000 µg.mL^{-1} TBZ	1.8de	1.2ef	-
Dip (1000 µg.mL^{-1} TBZ) and Wax (1.2 L.ton^{-1})	-	-	0.7ef
Dip (2000 µg.mL^{-1} TBZ) and Wax (1.2 L.ton^{-1})	0.5f	0.7ef	-

[a]Means followed by the same letter do not differ significantly.

Table 4.

Mean percentage infection (%) on fruit following curative and protective carnauba wax coating treatments of Valencia, Clementine and Satsuma fruit and inoculation with a TBZ-sensitive or TBZ-resistant isolate of *Penicillium digitatum*. Treatments consisted of coating fruit at three different wax loads (0.6, 1.2 or 1.8 L.ton^{-1}) incorporated with a TBZ concentration of 0 or 4000 µg.mL^{-1}. Fruit were incubated at 22°C for 4-5 days before infection was recorded and infection (%) calculated.

	Percentage infection (%)[a]					
	Satsuma		Clementine		Valencia	
Treatment	**Curative**	**Protective**	**Curative**	**Protective**	**Curative**	**Protective**
Sensitive isolate						
Control	98.2g	91.1a-e	90.1d-g	90.9e-g	82.8f-l	89.6k-n
Wax 0.6 L.ton^{-1}	94.8c-g	92.2b-f	84.1c-e	85.4c-e	73.7de	93.8mn
Wax 1.2 L.ton^{-1}	96.6fg	96.6fg	95.8g	91.1e-g	79.9e-i	91.1l-n
Wax 1.8 L.ton^{-1}	92.4c-f	96.4e-g	92.7fg	83.3cd	90.4l-n	94.8n
TBZ wax 0.6 L.ton^{-1}	95.3d-g	90.1a-d	89.8d-g	87.8c-f	40.4c	14.1a
TBZ wax 1.2 L.ton^{-1}	92.2b-f	86.7a	90.4d-g	67.2b	26.8b	19.8ab
TBZ wax 1.8 L.ton-1	94.0c-g	87.0ab	81.5c	58.9a	27.1e-h	19.5ab
Resistant isolate						
Control	97.4fg	96.6fg	93.8fg	94.5fg	79.4e-h	89.3j-n
Wax 0.6 L.ton^{-1}	-	-	-	-	80.5e-i	86.7h-n
Wax 1.2 L.ton^{-1}	-	-	-	-	80.7e-j	88.5i-n
Wax 1.8 L.ton^{-1}	-	-	-	-	85.4g-m	81.2e-k
TBZ wax 0.6 L.ton^{-1}	93.7c-g	95.1c-g	94.8fg	81.8c	68.5d	85.2g-m
TBZ wax 1.2 L.ton^{-1}	94.8c-g	95.1c-g	93.2fg	81.3c	75.0d-f	76.8d-g
TBZ wax 1.8 L.ton^{-1}	89.8a-c	95.6e-g	87.8c-f	84.1c-e	78.1e-h	86.7h-n

[a]Means with the same letter do not differ significantly

Table 5.

Mean sporulation incidence (%) following wax treatments on Valencia, Satsuma and Clementine fruit that were inoculated with either a sensitive or resistant isolate of *Penicillium digitatum* and treated curatively or protectively with a wax coating amended with 0 or 4000 µg.mL^{-1} TBZ applied at 0.6, 1.2 or 1.8 L.ton^{-1}; fruit were incubated at 22°C for 10 days before sporulation was rated.

	Sporulation incidence (%)[a]					
Citrus type	**Satsuma**		**Clementine**		**Valencia**	
Treatment	**Curative**	**Protective**	**Curative**	**Protective**	**Curative**	**Protective**
Sensitive isolate						
Control	99.0m	99.0m	99.0m	97.9lm	97.6lm	100.0m
Wax 0.6 L.ton^{-1}	100.0m	100.0m	100.0m	96.8lm	96.8lm	75.0g-i
Wax 1.2 L.ton^{-1}	99.0m	99.0m	100.0m	100.0m	100.0m	100.0m
Wax 1.8 L.ton^{-1}	100.0m	99.0m	97.6lm	94.7lm	100.0m	100.0m
TBZ wax 0.6 L.ton^{-1}	81.9ij	95.7lm	98.9m	100.0m	61.1fa	43.6e
TBZ wax 1.2 L.ton^{-1}	22.8c	54.8f	41.7e	73.7gh	30.5d	3.4ab
TBZ wax 1.8 L.ton^{-1}	0.0a	8.7b	0.0a	12.6b	9.2b	8.3ab
Resistant isolate						
Control	99.0m	100.0m	97.9lm	100.0m	82.1ij	100.0m
Wax 0.6 L.ton^{-1}	n/a	n/a	n/a	n/a	85.4jk	100.0m
Wax 1.2 L.ton^{-1}	n/a	n/a	n/a	n/a	77.1hi	97.8lm
Wax 1.8 L.ton^{-1}	n/a	n/a	n/a	n/a	82.1ij	100.0m
TBZ wax 0.6 L.ton^{-1}	100.0m	100.0m	100.0m	98.9m	69.5g	100.0m
TBZ wax 1.2 L.ton^{-1}	100.0m	100.0m	100.0m	95.7lm	81.2ij	80.0h-j
TBZ wax 1.8 L.ton^{-1}	100.0m	100.0m	100.0m	98.9m	91.7kl	100.0m

[a]Means with the same letter do not differ significantly

Table 6.

Mean chilling injury incidence on Clementine, Satsuma and Valencia fruit that were treated with a wax coating amended with 0 or 4000 µg.mL^{-1} TBZ applied at 0.6, 1.2 or 1.8 L.ton^{-1}, or dipped in a 1000 (Valencia) or 2000 (Clementine and Satsuma) µg.mL^{-1} TBZ suspension and then waxed with non-amended wax at 1.2 L.ton^{-1}; fruit were incubated at -0.5°C for 40 days and then at 22°C for 4 days before rated.

	Chilling injury incidence (%)[a]		
Treatment	**Clementine**	**Satsuma**	**Valencia**
Control	15.3hijkl	41.7bc	79.2a
Dip water	9.7kl	27.8defgh	64.6a
Dip TBZ	8.3kl	37.5bcdef	47.2b
Dip TBZ, wax: 1.2 L.ton^{-1}	4.2l	43.1b	18.1ghijk
Wax 0.6 L.ton^{-1}	28.1defgh	29.2cdefg	44.8b
Wax 1.2 L.ton^{-1}	14.6ijkl	47.9b	44.8b
Wax 1.8 L.ton^{-1}	4.2l	49.0b	38.5bcde
TBZ wax 0.6 L.ton^{-1}	13.5jkl	29.2cdefg	27.1efgh
TBZ wax 1.2 L.ton^{-1}	14.6ijkl	26.0fghi	22.9ghij
TBZ wax 1.8 L.ton^{-1}	9.4kl	39.6bcd	18.8ghijk

[a]Means with the same letter do not differ significantly.

Table 7.

Mean incidence of green buttons on Satsuma, Clementine and Valencia fruit that were treated with a wax coating amended with 0 or 4000 µg.mL^{-1} TBZ applied at 0.6, 1.2 or 1.8 L.ton^{-1}, or dipped in a 1000 (Valencia) or 2000 (Satsuma and Clementine) µg.mL^{-1} TBZ suspension and then waxed with non-amended wax at 1.2 L.ton^{-1}; fruit were incubated at -0.5°C for 40 days and then at 22°C for 4 days before being rated.

	Incidence of green buttons (%)[a]		
Treatment	**Satsuma**	**Clementine**	**Valencia**
Control	8.3j	77.8de	83.3bcde
Dip: Water	11.7ij	79.2de	58.3f
Dip: TBZ	16.7ij	84.7bcd	47.2fg
Dip and wax	13.9ij	93.1abc	56.9f
Wax 0.6 L.ton^{-1}	36.5gh	76.7de	79.4de
Wax 1.2 L.ton^{-1}	35.7h	96.9a	81.3de
Wax 1.8 L.ton^{-1}	37.5gh	96.9a	86.1bcd
TBZ wax 0.6 L.ton^{-1}	15.0ij	84.5bcd	85.4bcd
TBZ wax 1.2 L.ton^{-1}	20.4i	93.8ab	72.9e
TBZ wax 1.8 L.ton^{-1}	12.1ij	92.7abc	82.3cde

[a]Means with the same letter do not differ significantly.

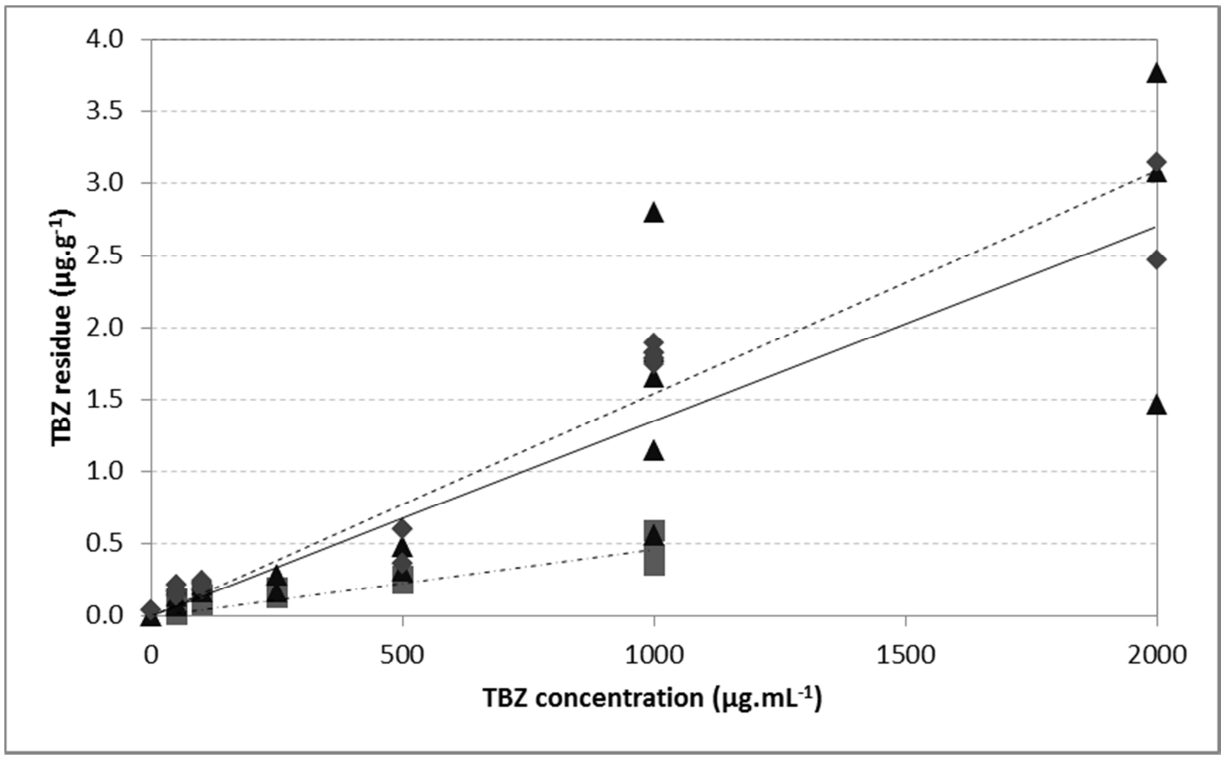

Figure 1. Mean thiabendazole (TBZ) residue levels and linear regression models fitted indicating trends of residue loading for Clementine (■, broken-dotted line; y = 0.0005x; R^2 = 0.77), navel (♦, broken line; y = 0.0015x; R^2 = 0.94) and Valencia (▲, solid line; y = 0.0014x; R^2 = 0.76) fruit that were dipped for 60s in TBZ suspension at concentrations ranging from 0 to 2000 µg.mL^{-1} at 22°C.

CHAPTER 3

CLASSIFICATION AND QUANTIFICATION OF IMAZALIL RESISTANCE IN *PENICILLIUM DIGITATUM*

Abstract

Soon after the introduction of imazalil (IMZ) as a postharvest fungicide on citrus, IMZ resistant isolates of *Penicilium digitatum*, the causal agent of green mould, were identified with varying frequencies in citrus packhouses around the world. Imazalil resistance is a quantitative trait with multiple genes involved. Most information is available on the *Cyp51* gene family, where resistance is caused by the insertion of DNA elements in the promoter region of the *Cyp51A* or *Cyp51B* genes, which results in overexpression of the genes. Based on the insertions in the promoter region, these isolates can be classified into three groups, R1, R2 and R3. The relative prevalence of these resistance groups in IMZ resistant *P. digitatum* populations is largely unknown in most citrus producing countries. In this study, the resistance group of 230 IMZ resistant *P. digitatum* isolates from green moulded citrus fruit from several countries (South Africa, the USA, Uruguay, Spain, Israel, Cyprus, Chile, Australia and Argentina) was determined using a published multiplex PCR assay. The resistance group of 189 isolates was successfully determined, but 41 of the isolates could not be classified into any of the three groups, indicating that other uncharacterised resistance groups might be involved in IMZ resistance in *P. digitatum*. It was also shown for the first time that some of the latter isolates contain the *Cyp51B* gene only. Isolates from the USA showed the highest resistance group diversity, with 13.7% of 73 isolates identified as R1, 12.3% R2 and 56.2% as R3, and 17.8% unclassified. From Chile, 1.5% of 67 isolates were R1, 76.1% were R3 and 22.4% were unclassified. All the isolates from the other countries were classified into the R3 group (83.3%) or were unclassified (16.7%). Due to the prevalence of the R3 group in all countries, a real-time PCR assay was developed for quantifying IMZ resistance in *P. digitatum* populations. The developed assay, which targets the promoter region involved in mediating resistance via the *Cyp51B* gene, was successfully optimized using mycelial DNA. However, quantification from spore suspensions could not be conducted reliably, as was observed for quantification of spores from filter paper spore traps and spore suspensions. Since previous reports showed that *Penicillium*-selective PDA-plate assays can be used for quantifying fungicide resistance in packhouses, this approach was also evaluated. The exposed plate assay was not reliable, since false positives were problematic for both of the evaluated selective media.

1. Introduction

Penicillium digitatum, the causal agent of citrus green mould, causes 90% of annual postharvest losses in citrus (Eckert and Eaks, 1989). Postharvest diseases are mainly controlled by synthetic fungicides (Korsten, 2006). The most widely used fungicides for green mould in South Africa are thiabendazole (TBZ) and imazalil (IMZ) (Dodd, 2010; Erasmus *et al.,* 2011).

Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-lH-imidazole; IMZ) is currently the most dependable fungicide for controlling green mould (Ladaniya, 2008). It is classified in the FRAC list of fungicides as code 3, which constitutes a medium risk for resistance development (FRAC, 2013). Imazalil is a sterol demethylation inhibitor (FRAC target site code: G1), (Hamamoto *et al.*, 2000), and is part of the imidazole fungicide group that inhibits ergosterol biosynthesis through the inhibition of the enzyme lanosterol 14α-demethylase (Siegel & Ragsdale, 1978). Ergosterol is a component of the fungal cell membrane that does not occur in higher eukaryotes. Lanosterol 14α-demethylase is therefore a good fungicide target since it selectively affects fungi but not higher eukaryotes such as plants and humans (Vanden Bosche, 1985). The amino acid sequence for lanosterol 14α-demethylase, which is encoded by the *Cyp51* gene family, is highly conserved but there are a few key differences among fungal species (Joseph-Horne and Hollomon, 1997; Becher and Wirsel, 2012). Fungal genomes also differ in the number of subtypes of *Cyp51*, with fungi belonging to the subphylum Pezizomycotina within the Ascomycota, containing one to three subtypes (Becher and Wirsel, 2012). Three *Cyp51* subtypes (*Cyp51A, Cyp51B* and *Cyp51C*) are known to occur in *P. digitatum*, which also belongs to the subphylum Pezizomycotina (Sun *et al.,* 2011).

Imidazole fungicides are classified as having a moderate risk of resistance development, and are also being researched intensely in the medical field, due to increasing problems with resistance (Brent and Hollomon, 1998; Becher and Wirsel, 2012). Soon after the introduction of IMZ to control TBZ-resistant isolates of *P. digitatum*, IMZ resistance developed and the number of resistant isolates continued to increase (Holmes & Eckert, 1999). Sánchez-Torres and Tuset (2011) found that 77% of their *P. digitatum* isolates that were collected in Spain were IMZ resistant. Holmes and Eckert (1999) found IMZ resistant isolates of *P. digitatum* in packhouses, but found that the level of resistance stayed constant over a 2-year period and suggested that this was due to the constant selection pressure in packhouses. They found that the EC_{50} levels of IMZ resistant isolates varied, depending on the age of inoculum used for the trial. Kinay *et al.* (2007) also found varying EC_{50} levels in IMZ resistant isolates. The number of resistant isolates seems to vary between locations and time frames. Kinay *et al.* (2007) found an IMZ resistance frequency of 86% in California, while Zhu *et al.* (2006) only found 2% in China. Seventeen percent was found by Bus *et al.* (1991) in Europe. Fogliata

et al. (2000) did a survey in Argentina and found only 5 isolates resistant to IMZ; only 3 of them were not controlled *in vivo* by 1000 µg.ml^{-1} IMZ. Low frequencies (1.5 and 0.1%) of resistant isolates were found in two packhouses in Brazil (Fischer *et al.,* 2009). In South Africa, IMZ-resistant *P. digitatum* isolates have been found, but the extent and levels of resistance is still unknown (Keith Lesar, pers. comm.).

IMZ sensitive isolates of *P. digitatum* seem to be generally more fit than IMZ resistant isolates (Dave *et al.,* 1989), but Holmes and Eckert (1995) found some IMZ resistant isolates that were more fit than sensitive isolates. This may be due to the overexpression of ATP-binding casette (ABC) transporter genes or efflux pumps that excrete both IMZ and host-defence compounds out of the fungal cell (Del Sorbo *et al.,* 1997; De Waard *et al.,* 2006; Nakaune *et al.,* 2002; Hulvey *et al.,* 2012; Wang *et al.,* 2012).

IMZ resistance is quantitative with multiple genes involved for several fungi, including *P. digitatum* (De Waard and Van Nistelrooy, 1990; Kanetis et al., 2010). Most is known about resistance involving the *Cyp51* gene family followed by ABC transporter genes. In *P. digitatum,* resistance involving the *Cyp51* gene family is characterized by the insertion of DNA elements within the promoter region of the genes. These insertions result in the overexpression of the genes and an IMZ resistance phenotype (Sánchez-Torres and Tuset, 2011). Three of the best studied IMZ resistance types, involving the promoter region of the *Cyp51A* or *Cyp51B* genes in *P. digitatum* have been characterised and are classified into three groups, i.e. R1, R2 and R3 (Sun *et al.*, 2011). The R1 resistance group contains a DNA element insertion of a 126 bp four-times tandem-repeated transcriptional enhancer into the promoter of *Cyp51A* (Hamamoto *et al.*, 2000). Isolates belonging to this resistance group were found in all 11 IMZ resistant isolates in a Japanese study (Hamamoto *et al.*, 2001), but only in three from 58 IMZ-resistant isolates in a Spanish study (Sánchez-Torres and Tuset, 2011). The R2 resistance group contains a DNA element insertion of 199 bp in the promoter region of the *Cyp51A* gene (Ghosoph *et al.*, 2007). The R3 resistance group also contains a 199-bp DNA element insertion in the promoter region, but this occurs in the promoter of the *Cyp51B* gene as opposed to the *Cyp51A* gene in the case of R2.(Sun *et al.,* 2011). This insertion element was later shown to be a miniature inverted-repeat transposable element (MITEs) that in the *Cyp51A* and *Cyp51B* promoters are almost identical, with 194 bp being similar and only the 5 bp target site duplication being different (Sun *et al.,* 2013a). This DNA element, referred to as PdMLE1, seems to be unique to *P. digitatum* and several copies (seven complete and 15 incomplete) of this insertion occur in the *P. digitatum* genome, with its frequency and location varying among isolates, but only leading to IMZ resistance when inserted into specific locations in the genome (Sun *et al.,* 2013b). The

R3 resistance group was the most widespread type of IMZ resistance in China; 89% of 297 resistant isolates had the R3 genotype. Although the *Cyp51C* gene is induced when IMZ sensitive isolates are treated with IMZ, its involvement in field resistance has not yet been proven (Sun *et al.,* 2011) and its involvement in IMZ resistance is still unclear.

Conventional and real-time PCR methods have been developed for identification and quantification of the R1 and R2 resistance groups in *P. digitatum*. Sun *et al.* (2011) developed a conventional multiplex PCR to identify R1, R2 and R3 resistance groups; the PCR targets the DNA insertion elements in the promoter regions of the *Cyp51A* and *Cyp51B* genes. Additionally, two real-time PCR protocols have been developed for specifically quantifying the R1 and R2 resistance groups (Chen *et al.,* 2008), but not the R3 group. The primers were shown to be species specific as well as resistance group specific using mycelial DNA. No attempt was made to optimize these qPCR assays for quantifying resistance in spores from environmental samples.

In addition to the *Cyp51* gene family, ATP-binding casette (ABC)-transporter genes have also been shown to play a role in IMZ resistance. Over-expression of the PMR1 and PMR5 genes are activated by the presence of toxicants and prevent accumulation of IMZ in the fungal cell (Sánchez-Torres and Tuset, 2011; Sun *et al.,* 2011). Recently, Sun *et al.* (2013b) found five additional ABC proteins similar to PMR1 and PMR5 that were also induced by IMZ treatment. The effect of these ABC transporters alone has not been shown to be sufficient to confer practical IMZ resistance.

The number of spores in a packhouse has a marked influence on disease control and the development of resistance to fungicides (Wild and Eckert, 1982; Kanetis *et al.,* 2007). A colony of *Penicillium* with a diameter of 2.5 cm may produce up to 4×10^8 spores (Kendrick, 1992). Bancroft *et al.* (1984) developed a potato dextrose agar (PDA) plate assay for monitoring *P. digitatum* fungicide resistance in packhouses. Smilanick and Eckert (1986) improved this method by adding selective compounds to the media. Exposed plates were placed in packhouses for a fixed period of time and the number of colonies counted and compared to the number of colonies on plates not amended with the fungicide, in order to quantify fungicide resistance.

Van Wyk (2011) preferred molecular quantification methods over exposed plate assays as PDA dishes dry out quickly and can only be used for short periods of time. Several pathogens have been quantified by real-time PCR from air samples (Fraaije *et al.,* 2002; Haugland *et al.,* 2004; Schweigkofler *et al.,* 2004), and other real-time PCR assays have been developed for detection of fungicide resistance (Bates and Taylor, 2001; Michalecka *et al.,* 2011). Real-time PCR assays are said to be very specific and reliable and contamination is prevented (Chevaliez *et al.,* 2012).

The aim of this study was to determine the relative frequencies of different IMZ resistance groups (R1, R2 and R3) involving the *Cyp51* gene family in South African *P. digitatum* populations, since no information is currently available on this. The prevalence of these groups was also investigated in isolates from other parts of the world (the USA, Uruguay, Spain, Israel, Cyprus, Chile, Australia and Argentina). Since the R3 resistance group was found to predominate among all of the evaluated resistant isolates, a real-time PCR methodology was developed to quantify this resistance group from environmental samples in packhouses. Lastly, conventional exposed plate assays using selective media were also evaluated for quantifying IMZ resistant isolates from packhouses.

2. Materials and methods

2.1. Isolate collection

A collection of 230 IMZ resistant *P. digitatum* isolates were kindly provided by Janssen PMP (Beerse, Belgium). The isolates were all obtained from green mould citrus fruits originating from various countries, including the USA (n = 75), Uruguay (*n* = 5), Spain (*n* = 23), Israel (*n* = 2), Cyprus (*n* = 2), Chile (*n* = 65), Australia (*n* = 4), Argentina (*n* = 13) and South Africa (*n* = 41). The IMZ EC_{50} values of these isolates were previously determined by Janssen PMP and were all >1 $\mu g.mL^{-1}$. An IMZ sensitive isolate (STE-U 6560), which was previously identified as *P. digitatum* (Erasmus *et al.,* 2011), was also included in the study.

2.2. Confirming the in vitro imazalil resistance phenotype of isolates

The IMZ resistance phenotype of all 230 isolates was confirmed by inoculating each isolate onto a non-amended PDA plate, a 0.5 $\mu g.mL^{-1}$ IMZ amended PDA plate and a 1.0 $\mu g.mL^{-1}$ IMZ amended PDA plate. An IMZ sensitive isolate (STE-U 6560) was also included as a control. Each plate was inoculated with one agar plug obtained from a pure culture growing on potato dextrose agar (PDA; DIFCO, Becton, Dickinson and Company, USA and Le Pont de Claix, France) media. The presence or absence of growth on plates was scored after 7 days of growth at 22°C.

2.3. Identification of IMZ Cyp51 resistance groups

The 230 *P. digitatum* isolates were classified into three different IMZ *Cyp51* resistance groups (R1, R2 or R3) by using the multiplex PCR assay method of Sun *et al.* (2011). Two IMZ sensitive isolates (STE-U 6560 and BLC38) was also included as controls. Template DNA for use in the multiplex PCR was obtained by first growing each isolate in 100 mL potato dextrose broth in 500-mL Erlenmeyer flasks for 10 days at 25°C in a shaking incubator (Labcon, Petaluma, CA, USA). After 10

days, the mycelia were harvested onto sterile cheese cloth and washed with sterile distilled water. The washed mycelia was placed into 2-mL Eppendorf tubes and stored at -20°C until DNA extraction was conducted. Genomic DNA was extracted from the mycelia using the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA) and a slight modification of the manufacturer's protocol. Glass beads (2 mm) were added to each 2-mL Eppendorf tube along with 400 µL lysis buffer. The tubes were shaken in a tissue lyser (MM 301, Retsch, Haan, Germany) for 10 min at maximum speed (30 Hz), and then incubated in a water bath at 65°C for 30 min. Tubes were centrifuged at 14 000 rpm for 8 min and the supernatant was processed according to the manufacturer's instructions. In the final step, the DNA was eluted from columns using 150 µL nuclease free water, and 1.2 µL RNAse was added followed by an overnight incubation step at 25°C. The DNA concentration and A260/280 ratios were determined with a Nanodrop (ND 1000, Wilmington, DE, USA).

The multiplex PCR reaction (Sun *et al.*, 2011) contained 1× buffer (Bioline USA Inc., Taunton, MA, USA), 0.7 U BIOTAQ DNA polymerase (Bioline USA Inc.), 0.25 mM of each dNTP , 0.1 µM of each primer (B1, B2, Cyp51A1 and Cyp51A2; Chen *et al.,* 2008; Sun *et al.,* 2011; Table 1), 0.002 mg BSA (Biowest Bovine Serum Albumin Lyophilised pH ≈7, Nuaillé, France), 3 mM $MgCl_2$ and 5 µL DNA in a final volume of 20 µL. Amplification was conducted on a GeneAmp PCR System 9700 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using 40 cycles of 95°C for 3 min, 95°C for 40 s, 56°C for 40 s, 72°C for 1 min and a final cycle of 72°C for 7 min. Amplification products were analysed on a 1% agarose gel that were stained with GR Green DNA Stain (Excellgen, Rockville, MD, USA). A 100-bp DNA ladder (Generuler ™, Fermentas Inc., Glen Burnie, MD, USA) lane was included in each gel in order to estimate the size of amplified PCR products.

Isolates that could not be amplified successfully, *i.e.* no amplification products were obtained, were re-amplified using less stringent PCR conditions: 1× buffer, 0.7 U BIOTAQ DNA polymerase, 0.25 mM dNTP , 0.4 µM of each primer (B1, B2, Cyp51A1 and Cyp51A2; Table 1), 0.002 mg BSA, 3 mM $MgCl_2$ and 4.5 µL DNA in a final volume of 50 µL. Amplification conditions consisted of 40 cycles of 95°C for 3 min, 95°C for 45 s, 54°C for 45 s, 72°C for 1 min and a final cycle at 72°C for 7 min.

2.4. Characterisation of P. digitatum *isolates that could not be classified into a IMZ Cyp51 resistance group*

Forty-one of the isolates could not be classified into any of the three resistance groups (R1 to R3) (see Results section 3.3). These isolates were further characterized for species identity, *in vivo* IMZ resistance and presence of the *Cyp51A* and *Cyp51B* genes.

2.4.1. Molecular species identification

The species identity of the isolates was confirmed using a published PCR restriction fragment length polymorphism (PCR-RFLP) assay that targets the β-tubulin gene in *Penicillium* (Glass and Donaldson, 1995). A known *P. digitatum* isolate (STE-U 2690) was included in the analyses as a reference. The PCR-RFLP method was conducted as described by Van der Walt *et al.* (2010) using the same template DNAs that were used in the multiplex PCR (Section 2.3). The PCR amplification reaction contained 0.2 µM of each primer (Bt2a and PentubR; Van der Walt *et al.,* 2010; Table 1), 0.2 mM of each dNTP, 1× PCR buffer (Bioline), 0.7 U BIOTAQ DNA polymerase (Bioline), 0.2 mg BSA (Biowest), 1.3 µl DNA, and 3 mM $MgCl_2$ in a final volume of 50 µL. Cycling conditions consisted of 40 cycles of 5 min at 94°C, 45 s at 94°C, 45 s at 55°C, and 60 s at 72°C and a final cycle of 7 min at 72°C. Amplified products were run on a 1% agarose gel containing GR green DNA stain (Excellgen) in order to confirm that amplification was successful prior to RFLP analyses.

RFLP analyses of the amplified PCR products were conducted using the restriction enzymes *Hae*III and *Rsa*I (Glass and Donaldson, 1995). The *Hae*III digest reaction consisted of 1× enzyme buffer (Fermentas Inc.), 1.5 µL *Hae*III (Fermentas Inc.), and 8 µL PCR product in a total volume of 25 µL. The *Rsa*I digestion reaction was similar, except that 0.5 µl *Rsa*I (Fermentas Inc.) was used. The restriction digest reactions were incubated overnight at 37°C, and 20 µL of the reactions were run on a 3% agarose gel that contained a 100 bp DNA ladder (Generuler ^{TM}, Fermentas Inc.) in the first and last gel lanes.

2.4.2. In vivo *IMZ resistance phenotype and pathogenicity determination*

The isolates were inoculated onto IMZ treated navel orange fruits to further confirm their resistance phenotype and pathogenicity. The isolates were grown on PDA for 7 days at 22°C, and 5 x 5 mm plugs were used to inoculate fruit that were washed with 1 $mL.L^{-1}$ didecyl dimethyl ammonium chloride solution (Sporekill, ICA International Chemicals, Stellenbosch, South Africa) and treated with 500 $\mu g.mL^{-1}$ IMZ for 60 s as a dip treatment. Each isolate was inoculated onto three control fruits and three IMZ treated fruit. After 7 days, fruit were evaluated for typical green mould symptoms, as well as the isolate's ability to grow on IMZ treated fruit, which characterised it as IMZ resistant. Previous studies have shown that IMZ sensitive isolates were not able to grow on IMZ-treated fruit (Erasmus *et al*., 2011, 2013; Njombolwana *et al*., 2013).

2.4.3. Amplification of the Cyp51A and Cyp51B genes

The presence of the *Cyp51A* and *Cyp51B* genes were determined through conventional PCR amplification using the same template DNA used in the multiplex (Section 2.3) and the β-tubulin (section 2.4.1) PCR amplifications. The presence of the *Cyp51A* gene was tested using a PCR reaction containing 0.2 µM of each primer (Pri717 and Pri1437c; Hamamoto *et al.,* 2000; Table 1), 0.32 mM of each dNTP, 1× PCR buffer (Bioline), 0.7 U BIOTAQ DNA polymerase (Bioline), 2 µL DNA, and 2 mM $MgCl_2$ in a final volume of 25 µL. Cycling conditions consisted of 40 cycles of 5 min at 94°C, 45 s at 94°C, 45 s at 57°C, and 45 s at 72°C and a final cycle of 5 min at 72°C. For the *Cyp51B* gene, published primers (CYP51B-F and CYP51B-R; Sun *et al.,* 2011) were not effective (results not shown) and new primers had to be designed using methods described for the R3 primer development (see section 2.6). The *Cyp51B* gene PCR reaction contained 0.2 µM of each primer (CYP51B-F1 and CYP51B-R1; Table 1), 0.32 mM of each dNTP, 1× PCR buffer (Bioline), 0.7 U BIOTAQ DNA polymerase (Bioline), 2 µL DNA, and 2 mM $MgCl_2$ in a final volume of 25 µL. Cycling conditions consisted of 40 cycles of 5 min at 94°C, 45 s at 94°C, 45 s at 57°C, and 45 s at 72°C and a final cycle of 5 min at 72°C. Isolates that did not yield any amplification products for the *Cyp51A* or *Cyp51B* gene were run with less stringent PCR conditions that consisted of the same reaction and amplification conditions, except that a higher primer concentration (0.4 µM) and a lower annealing temperature of 54°C were used.

2.5. Cluster analyses of IMZ EC50 values and their correlation with Cyp51 resistance groupings

The EC_{50} values were analysed by K-means cluster analysis (XLSTAT version 2013.5.09, www.xlstat.com) into 5 groups and correlated with resistance groups to ascertain any possible relationship between *Cyp51* resistance group and resistance level (MacQueen, 1967).

2.6. Development of a qPCR assay for quantifying IMZ resistance in packhouses

Two SYBR GREEN I chemistry based qPCR assays were developed: firstly, an assay to detect the R3 resistant group isolates only, and secondly an assay to detect both sensitive and IMZ resistant isolates. Four South African *P. digitatum* isolates (STE-U 2690, imb03, SR9-8 and SR-9-9) that belonged to the R3 resistance group and two IMZ-sensitive isolates (STE-U 6560 and BLC38) were sequenced using primers B1 and B2 (Sun *et al.,* 2011; Table 1). Sequences of two more isolates belonging to the R3 resistance group, Pdw03 (Accession number: HQ724323.1) and Pd1 (Accession number: GU124581.1) were obtained from Genbank. The sequence data were used to design the qPCR primers for the R3 resistance group using PrimerBlast (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Blast analyses of the primer sequences were run in Genbank to indicate their level of species specificity.

2.6.1. *Optimising the R3 qPCR using mycelial DNA.*

Three primer sets were designed and evaluated for quantifying the R3 resistance group, which targeted the PdMLE1 DNA insertion element in the promoter region of the *Cyp51B* gene and the promoter region upstream of this insertion. The primers were optimised and evaluated for species specificity using conventional PCR. The DNA that was used for determining species specificity was obtained from other closely related species and common fungi isolated from packhouses (Table 2). Primers qPdA1 and qPdA2 were selected for optimizing the qPCR assay using template DNA extracted from mycelia (See section 2.4.) from a R3 isolate (STE-U 2690). Primer annealing temperature was optimized by running a gradient qPCR with temperatures ranging from 52 - 62°C. The primer concentration was optimized by adding 50, 250 or 500 µM of each primer to the reaction. The $MgCl_2$ concentration was optimized by adding none, 0.75, 1 or 2 mM additional $MgCl_2$ to the reaction (the buffer contains 1.5 mM $MgCl_2$). The standard curve was drawn up by running a 4× dilution series of 20 $ng.\mu L^{-1}$ DNA in triplicate with 7 dilutions. The optimized assay was run with DNA from the sensitive isolate (STE-U 6560) as well as with that of other species (Table 2) to confirm the specificity of the qPCR assay. The qPCR reaction contained 1× SensimixSYBR (Bioline USA Inc., Taunton, MA, USA), 0.25 µM of each primer, 0.75 mM additional $MgCl_2$ and 2 µL DNA in a final volume of 20 µL. The qPCR was performed on a Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA, USA) using the following amplification conditions: 50 cycles of 95°C for 10 min, 95°C for 15 s, 62°C for 30, followed by melting curve analysis from 72°C to 95°C rising by 1°C and holding for 5 s after each step. All subsequent qPCRs were also run on the aforementioned Bio-Rad machine.

2.6.2. *DNA extractions from spore trap filter papers.*

The developed R3 resistance group specific qPCR assay was also evaluated for quantifying *P. digitatum* spores deposited onto filter papers, which resemble spore traps that have been used for quantifying *Fusarium* spores from air samples (Schweigkofler *et al.*, 2004; Van Wyk, 2011). The filter paper based spore traps and qPCR assays have the potential for quantifying *P. digitatum* from air sampled in the citrus packhouse environment. Firstly, a 10^7 spore suspension from isolate STE-U 6590 (R3 resistance group) was prepared. One hundred microliters of the suspension was pipetted onto a quarter filter paper (90 mm, Whatman no. 1, Germany), which was dipped in 1M TE buffer and fixed with Vaseline into a Petri dish in order to resemble the filter based spore traps (Schweigkofler et al, 2004; Van Wyk, 2011). The filter papers were left to dry overnight in a laminar flow cabinet. Each quarter filter paper was cut up and placed into a 2 mL Eppendorf tube. DNA was extracted from the filter papers using three different DNA extraction methods.

The first DNA extraction method consisted of the method reported by van Wyk (2011). Glass beads (2 mm) were added to each 2 mL tube along with 15 µL proteinase K (10 mg/L) and 1000 µL TES buffer [(20 ml 1 M Tris-HCL, 40 mL 0.5 M EDTA, 4 g sodium dodecyl sulfate (ICN Biomedicals, Ohio, USA)]. Tubes were vortexed for 1 min, sonicated for 1 min and were then shaken in a tissue lyser (Retsch) for 10 min at maximum speed. The tubes were placed in a water bath at 65°C for 1 h, followed by the addition of 270 µL NaCl (5 M) and 120 µL CTAB (10% per volume). The tubes were vortexed and incubated at 65°C for 10 min and centrifuged at 14 000 rpm for 30 min at 4°C. All subsequent centrifugation steps were also conducted at 14 000 rpm and 4°C. The supernatant (400 µL) was transferred to a new 1.5-mL tube and an equal volume of phenol:chloroform (1:1) was added, followed by vortexing and a 30-min centrifugation step. The supernatant (300 µL) was placed into a new tube and 400 µL phenol:chloroform (1:1) was added followed by vortexing. Tubes were then centrifuged for 15 min and 200 µL of the supernatant was placed into a new tube, followed by the addition of 400 µL chloroform, vortexing and centrifugation for 10 min. The supernatant (100 µL) was placed into a new tube and 60 µL of isopropanol added. Tubes were then placed in a freezer at -20°C for 15 hours followed by a 30-min centrifugation step in order to precipitate the DNA. The supernatant was discarded and the DNA pellet was washed using 60 µL of 70% ethanol and a 15-min centrifugation step. The DNA pellet was dried by placing the tubes into a heating block at 50°C. The dried pellet was re-suspended in 20 µL sterile distilled water and the DNA was stored at 4°C.

The second DNA extraction method made use of the Qiagen PlantMini kit (Qiagen, Limburg, Netherlands) and methodology according to manufacturer's instructions. The only exception was that in the first step, 1 mL of the AP1 extraction buffer along with 4 µL RNAse A and 0.3 g acid-purified sand (Sigma-Aldrich, St. Louis, MO, USA)were added to the filter paper in a 2 mL Eppendorf tube, which was then shaken in a tissue lyser (Retsch) for 5 min at 30 Hz speed. DNA was quantified using a Nanodrop and Qubit (Life Technologies), and the A260/280 ratios were noted.

A physical spore disruption DNA extraction method was also evaluated as published by Hu *et al.* (2014). Spores (2×10^7 suspension) were washed 3 times with sterile distilled water by centrifugation at 5000 x g for 5 min. The final spore pellet was re-suspended into 100 µL sterile distilled water and vortexed for 5 min with 1.5-mm Garnett beads (MO Bio, Carlsbad, CA, USA), kept in liquid nitrogen for 2 min, then kept at 65°C for 3 min and placed onto ice. The qPCR was run by adding 2 µL of the disrupted spore suspension to the reaction.

2.6.3. *DNA extractions from pure culture spore suspensions.*

Since DNA from the R3 isolate could not reliably be detected from spores deposited onto spore trap filter papers (see Results section 3.5), the real-time PCR was optimized from pure culture spore

suspensions. Spore suspensions for DNA extraction were obtained from 1 – 3 week old cultures growing on PDA. One hundred microliter spore suspensions were made up in Eppendorf tubes that contained 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 spores per tube. Three replications for each spore amount were made. The spore suspensions were lysed by first adding 0.3 g acid-purified sand (Sigma-Aldrich) to each tube, followed by shaking in a tissue lyser (Retsch) for 5×1 min at maximum speed. From this step onwards the Qiagen PlantMini kit protocol was followed according to manufacturer's instructions. DNA was quantified using a Nanodrop and Qubit (Life Technologies), and the A260/280 ratios were noted. The physical spore disruption method (Hu *et al.,* 2014) was also repeated on spore suspensions.

2.6.4. *Optimizing the R3 qPCR using DNA obtained from spore trap filter papers and pure culture spore suspensions.*

DNA extracted from the spore trap filter papers and spore suspensions was used to re-optimize the R3 qPCR assay that was described under section 2.6.1 for mycelia, to determine the sensitivity level of the assays and to compile standard curves. The R3-qPCR assay was re-optimized for primer annealing temperatures by running a gradient qPCR with temperatures ranging from 52 - 62°C. The primer concentration was optimized by adding 50, 300 or 900 µM of forward and reverse primers in all possible combinations to the reaction. Additional $MgCl_2$ was not added because it decreased specificity of the reaction. The reaction contained 1× SensimixSYBR, 0.9 µM of the forward primer (qPdA1), 0.3µM of the reverse primer (qPdA2), and 2 µL DNA in a final volume of 20 µL. Amplification conditions consisted of 60 cycles of 95°C for 10 min, 95°C for 15 s and 62°C for 30 s. A standard curve was drawn up using eight 4× serial dilutions of the DNA from the 10^7 pure culture spore suspension, and run along with DNA extracted in triplicate from the 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 spore suspensions. A standard curve was also constructed for the spore trap filter paper DNA in the same manner.

2.6.5. *Optimizing a qPCR for quantification of all* P. digitatum *isolates*.

The second qPCR assay, which detects both sensitive and all IMZ resistant group isolates, was optimized using primers T-F5 and T-R5, which bind in the coding region of the *Cyp*51A gene (Chen *et al.,* 2008; Table 1), and is *P. digitatum* specific primers. Primer annealing temperature was optimized by running a gradient qPCR with temperatures ranging from 52 - 62°C. The primer concentration was optimized by adding 50, 250 or 500 µM of each primer to the reaction. The standard curve was drawn up by running a 4× dilution series of 20 ng/µL DNA in triplicate with 7 dilutions. The qPCR reaction contained 1× SensimixSYBR, 0.2 µM of each primer and 2 µL DNA in a final volume of 20µL.

Amplification was conducted using 50 cycles of 95°C for 10 min, 95°C for 10 s, 60°C for 15 s and 72°C for 20 s. The assay was used to draw up a standard curve from R3 spores from filter paper spore traps (same as R3-qPCR).

2.7. Development of an exposed plate assay to quantify IMZ-resistance in packhouses

Media selective to *Penicillium* spp. were prepared according to the methods of Bancroft *et al.* (1984) and Smilanick and Eckert (1986). Bancroft medium contained PDA amended with neopeptone, dichloran and either IMZ (2 µg.mL^{-1}; Imazacure, 500 g.kg^{-1} SC, ICA International Chemicals, Stellenbosch, South Africa), or TBZ (10 µg.mL^{-1}; ICA Thiabendazole 500 SC, ICA International, Stellenbosch, South Africa). The TBZ plates were made to provide additional validation results. Smilanick medium contained the same ingredients as the Bancroft medium, but with the addition of o-phenylanizole (OPA) and pentachloronitrobenzene (PCNB) to the media. Eight drops of crystal violet (Sigma-Aldrich) were added per litre of media as colourant to the medium for better visualisation and counting of the young white *P. digitatum* colonies. Non-amended media was used as control plates. Plates were opened in packhouses and placed on a sterilised surface at least 1 m from the floor at the tip, wax coating applicator and packing areas for times ranging from 20 s to 15 min. Lids were placed face down on the sterilised surface. Plates were incubated at 22°C for 4 days before colonies were counted. This was done four times at three packhouses. Colonies were counted manually and by means of digital image analysis. Image analysis consisted of macrophotography of the open Petri dish after 4 days' incubation (Canon EOS 40D camera equipped with a 60 mm macro lens, fixed on a tripod). The white putative *Penicillium* colonies against the background of violet growth medium was identified and counted by image analysis software (Image Pro Plus software version 7.0; Media Cybernetics, www.mediacy.com). The number of colonies on an amended plate was divided by the number of colonies on the corresponding non-amended plate, and multiplied by 100 to obtain the percentage resistance frequency. To validate the method, 10 colonies were randomly picked from each non-amended plate, and placed on 10 IMZ amended PDA plates (2 µg.mL^{-1}) or 10 TBZ amended PDA plates (10 µg.mL^{-1}). Another 10 colonies were placed on non-amended PDA (incubation: 7 days). The percentage resistance was determined from these plates and compared to the percentage of resistance calculated with manual and image analysis counts. The 10 colonies growing on non-amended PDA was checked for typical morphological characteristics of *P. digitatum* (Raper and Thom, 1949) by viewing phialides and conidia under a microscope (Nikon Eclipse E600) at ×40 magnification.

3. Results

3.1. Confirming the in vitro *imazalil resistance phenotype of isolates*

All 230 isolates were able to grow on the 0.5 µg.mL^{-1} and 1.0 µg.mL^{-1} IMZ amended PDA plates (Figure 1), with the exception of the IMZ sensitive reference isolate STE-U 6560. The IMZ resistance phenotype of all the isolates from the collection was thus confirmed.

3.2. Identification of IMZ Cyp51 resistance groups

A total of 189 isolates yielded amplification products with the multiplex PCR and these isolates could be classified into resistance groups R1 to R3 according to their banding patterns as described by Sun *et al.* (2011) (Figure 2). The sensitive isolate yielded a banding pattern consisting of approximately 401 and 506 bp, which was distinct from groups R1 to R3 (Figure 2). The remaining 41 isolates did not yield any amplification products and could thus not be classified into a resistance group. Re-amplification of the 41 isolates using less stringent PCR conditions (See section 2.2), with different DNA concentrations, primer concentrations, annealing temperature and $MgCl_2$ concentrations (Section 2.2), only yielded non-specific amplification products.

Isolates from the USA showed the greatest resistance group diversity with 13.7% from 75 isolates identified as R1, 12.3% R2 and 56.2% R3 resistance group, and 17.8% unclassified (Table 3). In Chile, 1.5% R1, 76.1% R3 and 22.4 % unclassified isolates were found from 67 isolates tested. All the isolates from the other countries were classified into the R3 group (83.3%) or were unclassified (16.7%).

3.3. Characterisation of P. digitatum isolates that could not be classified into a IMZ Cyp51 resistance groups

The fourty-one isolates that did not yield any amplification products with the multiplex PCR of Sun *et al.* (2011) were characterised further for species identity, *in vivo* IMZ resistance and the presence of the *Cyp51A* and *Cyp51B* genes.

3.3.1. Molecular species identification

PCR-RFLP analysis of the β-tubulin gene region of the 41 isolates showed that they all had the banding pattern characteristic of *P. digitatum*, which corresponded to that of the *P. digitatum* reference isolate STE 6560 (Figure 3). The successful PCR amplification of the single copy β-tubulin gene region from all 41 isolates also showed that the genomic DNA quality of the extracted DNA of

these isolates were of amplifiable quality. The same genomic DNA was also used in the multiplex PCR resistance group PCRs.

3.3.2. In vivo IMZ resistance phenotype and pathogenicity determination

The 41 isolates showed typical green mould symptoms on untreated control fruit, as well as on the IMZ treated fruit. Some of the isolates also showed signs of sporulation inhibition on IMZ treated fruit (Figure 4).

3.3.3. Amplification of the Cyp51A and Cyp51B genes

The observation that 41 of the isolates yielded no amplification products with the multiplex resistance group PCR, prompted further analyses to ensure that the template genomic DNA was of good quality and that the isolates did contain the two *Cyp51* genes involved in IMZ resistance. Thirty seven of the isolates yielded the correct sized amplification product when amplified with the *Cyp51A* and *Cyp51B* gene primers, and thus contained both genes. Four of the isolates yielded amplification products with the *Cyp51B* gene primers only, and thus did not contain the *Cyp51A* gene (1 isolate from Argentina, 1 from Chile and 2 from USA). Re-amplification of the latter isolates using less stringent PCR conditions did not yield any *Cyp51A* amplification products. Amplification of the *Cyp*51*B* gene from all 41 isolates further confirmed that the genomic template DNA used in the multiplex PCR was of amplifiable quality.

3.4. Cluster analyses of IMZ EC_{50} values and their correlation with Cyp51 resistance groupings

Cluster analysis of EC_{50} values revealed five different classes among the 189 isolates analysed. The centroids were 1.29, 1.77, 2.32, 6.37 and 13.56 $\mu g.mL^{-1}$ for class 1, 2, 3, 4 and 5, respectively. Isolates from the R3 resistance group occurred in all five classes, while R1 and R2 isolates occurred in classes 1, 2 and 3 only (Figure 5). No pattern could be observed in cluster analysis by country; isolates from most countries occurred in all classes, except for class 5 which only contained isolates from Cyprus, Chile and Spain (results not shown).

3.5. Development of a qPCR assay to quantify IMZ resistance in packhouses

3.5.1. Optimising the R3 qPCR using mycelial DNA.

Conventional PCR evaluation of the three primer pairs designed for quantification of the R3 resistance group showed that primer pair qPdA1 and qPdA2 was species specific to *P. digitatum,* since it did not amplify DNA from other species (Table 2). The other two primer pairs were not

species specific. The qPdA1 and qPdA2 primer pair was further evaluated and optimised for use in the R3 qPCR using DNA obtained from mycelia. The quality of all qPCR amplifications was measured by the ability to draw up a standard curve, which met the following requirements: an efficiency of 90 – 100%, a R^2-value of > 99%, and a slope (m-value) of -3.6 to -3.1 (Fraga *et al.,* 2008). The y-intercept was used as a general indication of the probability to use the standard curve in subsequent sample analysis: y-intercepts above 30 cycles were regarded as indicative of less accurate assays (Burns *et al.,* 2008). qPCR using DNA obtained from mycelia as template yielded a standard curve with an m-value of -3.487, efficiency of 94% and R^2-value of 99.9%, which was acceptable.

3.5.2. Optimising the R3 qPCR using DNA obtained from spore trap filter papers.

The R3 qPCR analyses from spore filter papers (spore traps) were able to detect the R3 resistance group isolates, but the analyses were not reliable due to the inability to draw up good quality standard curves from all three evaluated DNA extraction methods since their efficiency-, R^2- and m-values were unacceptable. The values for the standard curve of the Van Wyk (2011) DNA extraction method could not be determined, since the highest spore concentration only amplified after 48 cycles. For the Qiagen kit DNA extractions, the efficiency was 110.0%, m-value -2.912 and R^2 - 77.2%. For the physical disruption DNA extraction method a standard curve could not be drawn up as all three replications of each spore concentration could not be amplified.

This could have been due to poor quality DNA extractions from the spore filter papers for all three DNA extraction methods, as well as very low DNA concentrations. DNA extractions from spore filter papers using the method of Van Wyk (2011) did not yield good A260/280 ratios (2.40 – 2.59), since these were markedly poorer than the recommended ratio of 1.8 (Fredlund *et al.,* 2008). The lowest quantification cycle (C_q) obtained in qPCRs using this DNA was 48. DNA extractions from filter paper samples without Vaseline using the Van Wyk (2011) DNA extraction method had better A260/280 ratios (1.40 – 1.51), but still had a very low DNA yield (6.12 – 10.30 $ng.\mu L^{-1}$) and could therefore not be quantified accurately. DNA yield from the Qiagen kit extractions were extremely low (< 1.34 $ng.\mu L^{-1}$), and the A260/280 ratios (1.15 – 1.26) were also poor.

3.5.3. Optimizing the R3 qPCR using DNA obtained from pure culture spore suspensions.

The use of the R3 resistance qPCR for quantification of DNA obtained from a concentration range of pure culture spore suspensions was not reliable due to the unacceptable values obtained for the standard curves for both DNA extraction methods. The Qiagen kit extraction method yielded an efficiency of 96.2%, R^2 of 72.7% and m-value of -3.416. The physical disruption method did not

yield a standard curve since the highest spore concentration was only detected after 40 cycles, and not all three replicates of each dilution was amplified.

The inability to obtain acceptable standard curves could also have been due to low quality DNA and low DNA concentrations, which were A260/280 ratio of 0.50 - 0.62 and DNA concentration of 2.5 ng/µL for 10^7 spores for the Qiagen extraction method. The physical spore disruption suspensions was not quantified due to the fact that it was a crude DNA extraction method that cannot be quantified with the Nanodrop. The C_q for DNA extracted using the Qiagen DNA extraction method was 35.41 cycles (y-intercept of the standard curve), which corresponded to more than 9765 spores. Spores could not be accurately quantified according to the melting curve analysis, since qPCR on extractions from suspensions with less than 9765 spores amplified products with different melt peaks. The assay also detected 10^5 IMZ-sensitive spores only after a C_q of 38, although its product yielded a slightly different melt peak (81°C *vs.* 84°C, for resistant and sensitive respectively). Intra-assay variability was very high and unacceptable.

3.5.4. Optimizing a qPCR for quantification of all P. digitatum *isolates.*

The second qPCR assay, using primers T-F5 and T-R5 (Chen *et al.,* 2008; Table 1), which are *P. digitatum* specific primers, was close to being optimised for mycelial DNA, but since it did not have the precise correct efficiency (88%), m-value (-3.64) or R^2-value (99.8%), it is not surprising that the assay was not successful when using DNA extracted from spores. After it was found that not all isolates contain the *Cyp*51A gene (see Section 3.4.3.), which is the annealing site of these primers, the further development of the assay was abandoned.

3.6. Development of an exposed plate assay to quantify IMZ-resistance in packhouses

The exposed plate assay with the Bancroft and Smilanick media was evaluated 4 times during the 2011 season at 3 sites (tip, wax and pack stations) in three commercial packhouses. In general, exposed plates at the tip stations yielded more colonies than wax and pack stations (from 10 times to 250 times more, depending on the specific day sampled; results not shown). *Penicillium* colonies on Bancroft media grew faster and more colonies were observed than on Smilanick media. It was found that opening plates for 5 min were sufficient, as longer exposure times often led to more than 300 colonies on a plate. The frequencies of putative IMZ and TBZ resistant isolates counted manually, correlated well with the frequencies determined by image analysis (Figure 6), but the TBZ and IMZ resistance frequencies calculated from re-isolations of *P. digitatum* correlated very poorly with those determined by manual counts (Figure 7 and 8). Also, re-isolations from plates containing the Bancroft or Smilanick media revealed that in some packhouses, up to 80% of white colonies

were not *P. digitatum*, but *Alternaria*, *Cladosporium* and *Epicoccum*. A large number of yeast colonies that were found to be antagonistic to *P. digitatum* were trapped by and isolated from the selective Bancroft and Smilanick media. As these yeast colonies occurred more frequently on fungicide amended plates compared to unamended control plates, its presence could not be ignored. Due to the low specificity of the media and common presence of antagonistic yeasts, which influenced the results, further evaluation and development of the exposed plate assay was abandoned.

4. Discussion

The current study contributed significantly to our knowledge on IMZ resistance mechanisms in *P. digitatum* populations, and the molecular quantification of resistance using qPCR. It was shown that the R3 resistance group is most prevalent in most citrus producing countries including South Africa, and that a qPCR assay targeting this resistance group thus has the potential for high throughput molecular IMZ resistance quantification in packhouses. Isolates belonging to the R1 and R2 resistance groups were only detected at very low frequencies in the USA and Chile.

The study developed a qPCR for quantifying the R3 resistance group, but further work is required on DNA extraction methods that are sufficient for reliable quantification of spores from filter paper spore traps and pure culture spore suspensions. A significant finding of the study, which also complicates the use of the R3 resistance qPCR, was the presence of isolates (22% of all evaluated isolates) that did not contain any of the known mutations or insertions associated with the *Cyp51* gene family resistance groups. The resistance mechanism present in these isolates still needs to be elucidated, and if it is found to be similar in all isolates, a qPCR targeting this resistance group will be beneficial as it predominated over the R1 and R2 groups. Since quantification of IMZ resistance using qPCR might be challenging, a conventional plate assay for quantifying resistance was also evaluated. This assay was not deemed suitable for IMZ resistance quantification since several false positives were detected using two different selective mediums.

One of the main aims of this study was to develop a qPCR assay for detecting and quantifying IMZ resistant spores, as well as sensitive and resistant *P. digitatum* spores, in order to be able to determine an IMZ resistance frequency based on the outcomes of the two assays. This required information on the mechanisms, and more specifically the marker genes associated with IMZ resistance in *P. digitatum*. This was obtained by grouping isolates for resistance mechanism involving the *Cyp51* gene family that was characterised in *P. digitatum*. Resistance classification using a multiplex PCR (Sun *et al.*, 2011) revealed that most isolates (average 78%) from each country

belonged to the R3 resistance group that contains a 199-bp MITE-like DNA element (PdMLE1) in the promoter region of the *Cyp51B* gene, which causes overexpression of the gene and consequently a IMZ resistance phenotype (Sun *et al.,* 2011). Sun *et al.* (2011) also found a great number (89%) of R3 isolates in China, thus confirming the prevalence of this resistance group and its suitability for use as a target to developing a qPCR assay to quantify IMZ resistance. The R3 resistance group qPCR assay was successfully optimised for DNA isolated from mycelia. However, the qPCR could not be optimised for DNA extracted from filter paper spore traps. It was found that DNA isolated from these spore traps using three different DNA extraction methods was of low quality, based on A260/280 ratios values, and not useful for qPCR analyses. Other studies have, however, been able to use filter paper spore traps very successfully for quantification of *Fusarium* spp. spores in air samples (Schweigkofler *et al.,* 2004; Van Wyk, 2011). This might be due to the larger size or cell wall composition of *Fusarium* spores compared to *Penicillium* spores and differences in inhibitor substances in the DNA extractions.

Since quantification from spore trap filter papers was not successful, DNA extraction and qPCRs from pure culture spore suspensions was attempted as a first step to develop a successful qPCR assay from the spore filter papers. However, this also proved difficult since an acceptable standard curve could not be generated based on too low R^2 values. This probably also resulted in the low detection limit of 10000 spores (C_q of 35.41) that will not be useful for detecting resistance from environmental samples. Of some concern was the observation that IMZ-sensitive spores were detected at a C_q (y-intercept) of 38.94 in the pure culture spore suspension qPCRs. However, melt curve analysis showed that the product from the sensitive isolate could be differentiated from the R3 resistance group melt curve since it had a slightly higher peak melting temperature. Detection of the sensitive isolate might be explained by the fact that the reverse primer binds to the PdMLE1 DNA element, of which there are at least 22 copies in the *P. digitatum* genome, although all copies might not be full length copies (Sun *et al.,* 2013b). R3 detection might therefore be more successful with a reverse primer that is more specific to the *Cyp51B* 5' gene region. As the primers should also be species specific, it might be challenging to design primers flanking the insertion site in the *Cyp51B*-gene region. The use of a probe assay that contains a probe that binds internal to the two currently used primers might help to overcome this problem.

The greatest challenge of the qPCR assay development was to extract both good yield and good quality DNA from pure culture spore suspensions and filter paper spore traps. Quantification using qPCR should be more accurate with a higher DNA yield, or primers that pick up 10^7 spores before a C_q of 30, which is generally accepted as the cut-off point for reliability of a qPCR assay (Burns *et al.,*

2008). Higher DNA yield might be obtained by optimising the lysing method; for example, using different types and combinations of lysing beads (glass, ceramic ect.) and lysing times. Haugland *et al.* (2004) were able to develop a successful Taqman qPCR using DNA extracted from *Penicillium* spores with a Qiagen kit as described by Haugland *et al.* (2002). Their limit of detection for *P. digitatum* spores was, however, also relatively high (4×10^3), but still better than that of the R3 qPCR. This is most likely due to the fact that their qPCR targeted the rDNA region that is present as 100s of copies in fungal genomes (Borsuk *et al.,* 1982), as opposed to the *Cyp51B* gene that is a single copy gene. Thus, in general it seems that it is difficult to extract DNA and quantify DNA from *Penicillium* spores, perhaps due to the size and composition of the spores and inhibitors.

Not all IMZ isolates genotyped in this study could be classified into the R3 resistance group using the multiplex PCR of Sun *et al.* (2011) or any of the known *Cyp51* resistance groups, since 41 isolates (22%) did not yield any amplification products with the multiplex PCR. This lack of amplification was not due to bad quality genomic DNA, since the genomic DNA was amplified in PCRs targeting the β-tubulin region (PCR-RFLP) and the *Cyp51B* gene. Using less stringent multiplex PCR conditions also did not yield any amplification products, thus showing that novel IMZ resistance mechanism/s are most likely involved in the 41 isolates. It was exceptional that the 41 isolates did not yield any amplification products with the multiplex PCR, since this PCR does yield products for IMZ sensitive isolates due to the annealing of the two primer pairs in the promoter regions of the *Cyp51A* or *Cyp51B* genes and the respective 5' region of the genes. Size differences in PCR amplification products between IMZ sensitive and resistant group isolates are evident due to the insertion of DNA elements in the promoter region of the genes. The complete lack of amplification products in the 41 isolates could thus be due to mutations or deletions in the primer binding sites in the promoter region and/or 5' gene regions of the *Cyp51A* and *Cyp51B* genes. The latter was shown to be a possibility in four isolates, since these isolates only contained the *Cyp51B* gene based on PCR amplifications. This would, however, have to be confirmed using Southern analyses. Thus far, all fungi belonging to the Pezizomycotina have been shown to contain the *Cyp51B* whereas some may lack the *Cyp51A* gene (Becher and Wirsel, 2012). In order to determine whether deletions/mutations in the *Cyp51A* or *Cyp51B* promoter regions or genes may be the cause of the novel resistance genotype/s, sequencing of the genes and their promoter regions will have to be conducted in future. This will require the development of more universal primers in conserved regions of the promoter area of the *Cyp51A* and *Cyp51B* genes. Although mutations within the *Cyp51* genes themselves seem unlikely to be the cause of resistance, since resistance has only been linked to alterations in the promoter region for *P. digitatum*, this must be investigated since in other fungi DMI resistance is linked to the coding region of the *Cyp51* genes itself (Wyand and Brown, 2005; Leroux *et al.,* 2007).

It cannot be ruled out that the IMZ resistance in these isolates might be caused by ABC transporters or the *Cyp51C* gene, but currently there is no evidence that these genes contribute to practical levels of IMZ resistance by themselves. ABC transporters may rather modulate the level of resistance in *Cyp51* gene mediated resistance as seen in variable EC_{50} values. If it is found that the annealing sites of the multiplex PCR primers have mutations that does not necessarily lead to resistance, it is possible that completely novel genes and their promoters might be involved in these isolates; this could best be investigated using whole genome sequencing.

There was no correlation between the level of resistance (measured as EC_{50} values) of isolates and whether they were the R1, R2 and R3 genotype. Isolates from USA showed the highest EC_{50} values, as well as the greatest diversity in genotypes among isolates. It is unknown whether difference in genotype leads to different fitness penalties, virulence and levels of resistance. Dave *et al.* (1984) certainly observed that some IMZ resistant isolates were less fit than others, and it would be interesting to see whether this can be linked to the genotype, or even to other resistance genes. Since this study found such a small number of R1 and R2 isolates, the possibility that genotype plays a role in the level of resistance (e.g. EC_{50}) of isolates, cannot be completely ruled out.

Conventional resistance quantification methods can be highly laborious as sufficient numbers of single isolates need to be collected and tested for sensitivity. For *P. digitatum*, conventional methods are even more laborious given this fungus's profuse sporulation habits in culture (Kendrick, 1992). As a consequence, previous researchers have developed exposed plate assays to measure and quantify fungicide resistance in *P. digitatum* (Bancroft *et al.,* 1984; Smilanick and Eckert, 1986). However, the exposed plate assay proved to be unreliable in our assessments, and there was no correlation between colony counts on the selective media and re-isolations on PDA. The media was not solely selective to *Penicillium* spp. growth, and genera could not be differentiated after 4 days' incubation. In certain cases yeast colonies antagonistic to *P. digitatum* occurred on unamended and fungicide amended plates (and more commonly on fungicide amended plates), which affected the outcome of the assessment. For this reason, it is also not feasible to incubate the plates for longer so that one can distinguish between *Penicillium* spp. and other colonies. Also, plates could not be left open for longer than 5 min, which means that a large number of sampling will have to be done to get an accurate idea of the spore load in a packhouse. Bancroft *et al.* (1984) also observed highly variable results using this method, and they consequently could not observe statistically significant differences among treatments.

In conclusion, this study proved the concept of a qPCR assay to detect and quantify IMZ resistance in *P. digitatum* populations, but further development is required to realise the assay's

practical potential. It is possible to develop highly specific and sensitive molecular assays for monitoring inoculum, but primer design is very important and when more restrictions are imposed upon primers (*i.e.* it should be highly sensitive, species-specific and resistance group specific) it can be challenging to find primers that perform well in qPCR. Even after a molecular technique has been developed, it is not fool proof and may lead to false negatives, as was demonstrated by the multiplex PCR used to genotype isolates.

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Table 1. Sequences of primers used in this study.

Primer	Sequence (5' - 3')	Reference
B1	TATAGCGACATTAGTTTGGC	Sun *et al.,* 2011
B2	AGGAAAGTTGCAGAGAGACCCAT	
CYP51A1	TAGCTCCAAAACAAATCGTCTGCC	Chen *et al.,* 2008
CYP51A2	GGTGAAGATATTGCCGTACTAGAC	
T-F5	CGCATACTATACTACTGGACTTG	Chen *et al.,* 2008
T-R5	GTCTTGTTATTTGGAAAGTGTTAG	
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Van der Walt *et al.,* 2010
PentubR	GACGGACGACATCGAGAACCTG	
Pri 717	CTCCAAGGCGAAGAAGTACGATCA	Hamamoto *et al.,* 2000
Pri1437c	CCGTATCAGAAGAATCCTCGGC	
CYP51B-F1	CACTGCTCGACCCAGTCAAT	Designed for this study
CYP51B-R1	ACTCCACGTTTCTCGTACCG	
qPdA1	AAGGGGCGGGTCTCTCGCCG	Designed for this study
qPdA2	TGTCTCGGCATGACGCCATTGAGGC	

Table 2. Species and isolate numbers of isolates used to test primer specificity of primers designed for the R3 qPCR assay to quantify IMZ resistance in *P. digitatum*.

Isolate	Species
STE-U 6592	*Alternaria alternata* pv. *citri*
STE-U 6593	*Alternaria alternata* pv. *citri*
STE-U 6813	*Colletotrichum gloeosporioides*
STE-U 6814	*Geotrichum citri-aurantii*
STE-U 7649	*Penicillium brevicompactum*
STE-U 7647	*Penicillium brevicompactum*
STE-U 7651	*Penicillium brevicompactum*
STE-U 7644	*Penicillium chrysogenum*
STE-U 7646	*Penicillium chrysogenum*
STE-U 7648	*Penicillium chrysogenum*
STE-U 7639	*Penicillium crustosum*
STE-U 7645	*Penicillium crustosum*
STE-U 7650	*Penicillium crustosum*
STE-U 7638	*Penicillium expansum*
STE-U 7640	*Penicillium expansum*
STE-U 7641	*Penicillium expansum*
CV 1181	*Penicillium glabrum*
CV 36	*Penicillium glabrum*
STE-U 7643	*Penicillium glabrum*
STE-U 7406	*Penicillium italicum*
STE-U 7408	*Penicillium italicum*
STE-U 7411	*Penicillium italicum*
STE-U 7642	*Penicillium italicum*
STE-U 6378	*Phytopththora citricola*
STE-U 6558	*Phytopththora citricola*
STE-U 6815	*Trichoderma* spp.
STE-U 6816	*Trichoderma* spp.
STE-U 6818	*Trichoderma* spp.
Disease clinic isolate*	*Cladosporium* spp.
Disease clinic isolate	*Rhizopus* spp.
Disease clinic isolate	*Epicoccum* spp.

*Isolates identified to genus level that were obtained from University of Stellenbosch, Plant Pathology Disease Clinic.

Table 3. Percentage of *P. digitatum* isolates obtained from green moulded citrus fruit from different countries from each genotype (R1, R2, R3 or unclassified) as determined by a conventional multiplex PCR developed by Sun *et al.* (2011), and the number of isolates used from each country.

	Isolates (%)				Number of
Country	**R 1**	**R 2**	**R 3**	**Unclassified**	**isolates used**
Argentina	0.0	0.0	61.5	38.5	13
Australia	0.0	0.0	75.0	25.0	4
Chile	1.5	0.0	76.1	22.4	67
Cyprus	0.0	0.0	100.0	0.0	2
Israel	0.0	0.0	100.0	0.0	2
South Africa	0.0	0.0	94.3	5.7	35
Spain	0.0	0.0	90.9	9.1	23
Uruguay	0.0	0.0	40.0	60.0	5
USA	13.7	12.3	56.2	17.8	73

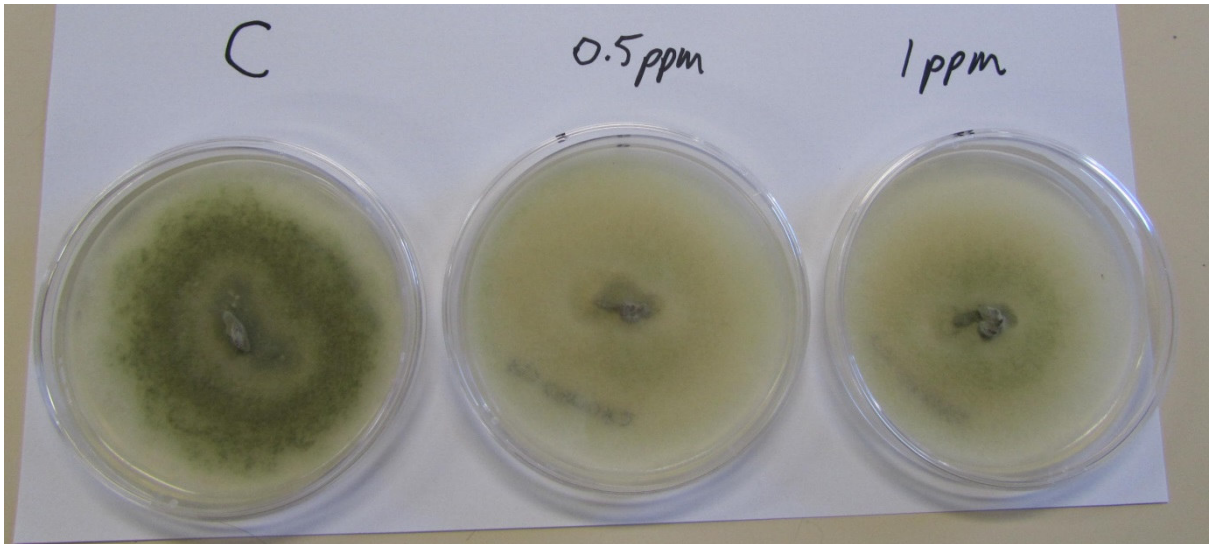

Figure 1. A *Penicillium digitatum* isolate tested for IMZ sensitivity. Left dish: Control plate, Middle dish: 0.5 µg.mL^{-1} IMZ amended, and right dish: 1 µg.mL^{-1} IMZ amended.

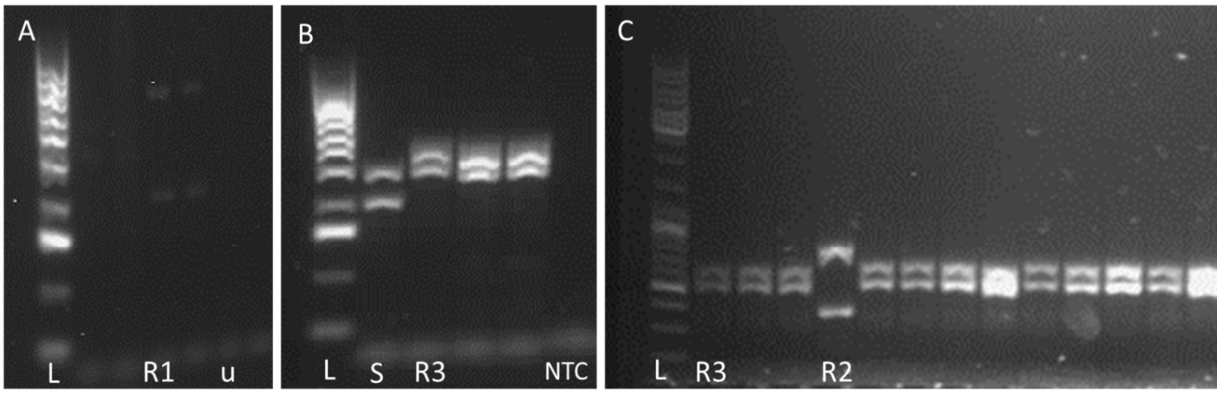

Figure 2. Agarose gels containing amplification products of *Penicillium digitatum* DNA amplified with a multiplex PCR (Sun *et al.,* 2011) that differentiates *Cyp51* gene associated imazalil resistance groups. The banding patterns of the R1 (401 and 1010 bp) and unclassified (u) groups (A); banding patterns of R2 (401 and 705 bp) and R3 (506 and 600 bp) groups (B); and banding pattern of the sensitive isolate (401 and 506 bp; S) and R3 group (C) are shown along the 100 bp DNA ladder indicated as L.

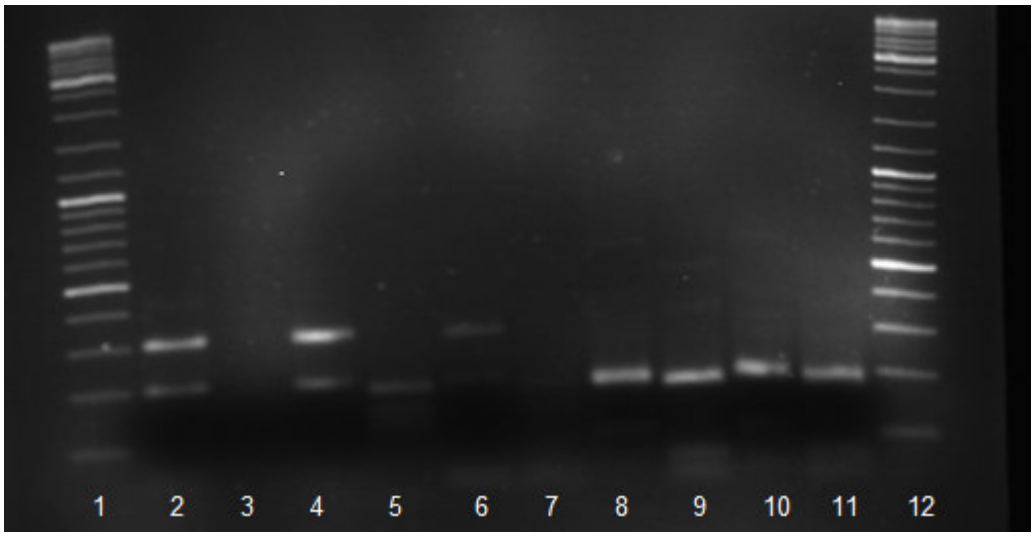

Figure 3. The polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) group that were identified for *Penicillium digitatum* (Lanes 8 – 9 and 10 - 11). The PCR-RFLP groups were identified by conducting *Rsa*I (even numbered lanes) and *Hae*III (odd numbered lanes) restriction digests on PCR amplified products of the partial β-tubulin gene. A 100-bp DNA ladder (Lanes 1 and 12) was run along with the digest products. Lanes 2 and 3: *Penicillium expansum*, Lanes 4 and 5: *Penicillium crustosum*, Lanes 6 and 7: *Penicillium italicum*.

Figure 4. Imazalil (IMZ) resistant isolates growing on 500 µg.mL^{-1} IMZ dip-treated fruit. The left and right lane show typical green mould symptoms, with the left lane showing sporulation inhibition by IMZ, and the middle lane shows a IMZ sensitive isolate. Fruit were incubated for 10 days at 22°C.

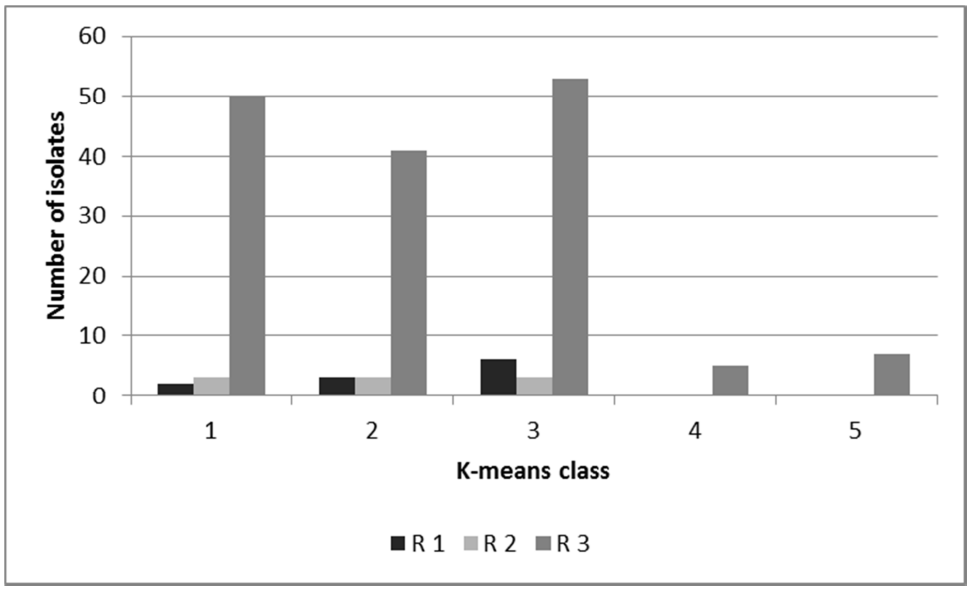

Figure 5. K-means cluster analysis of EC_{50} values of 189 *Penicillium digitatum* isolates that are IMZ-resistant and classified by multiplex PCR as either R1, R2 or R3 type resistance. The centroids for each class were 1.29, 1.77, 2.32, 6.37 and 13.56 µg.mL^{-1} for class 1, 2, 3, 4 and 5, respectively.

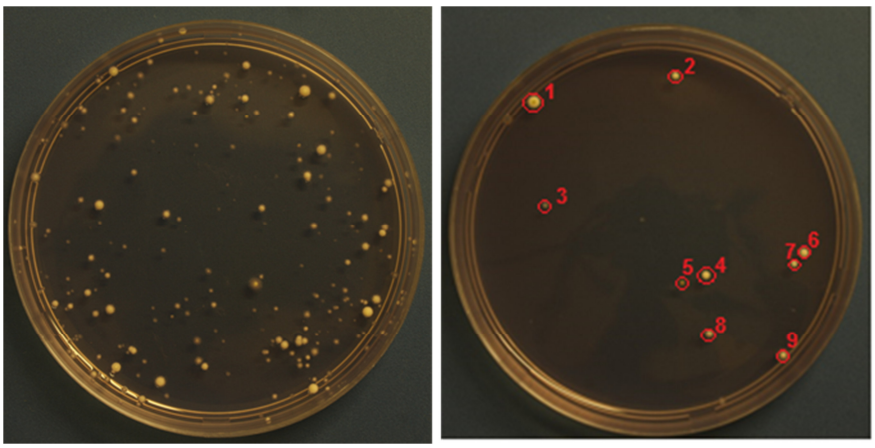

Figure 6. Exposed plate assay to quantify IMZ resistance in *P. digitatum.* The plate on the left is the unamended control plate, and the plate on the right is amended with 2 µg.mL^{-1} IMZ as counted by the image analysis software. Plates were incubated for 4 days at 22°C.

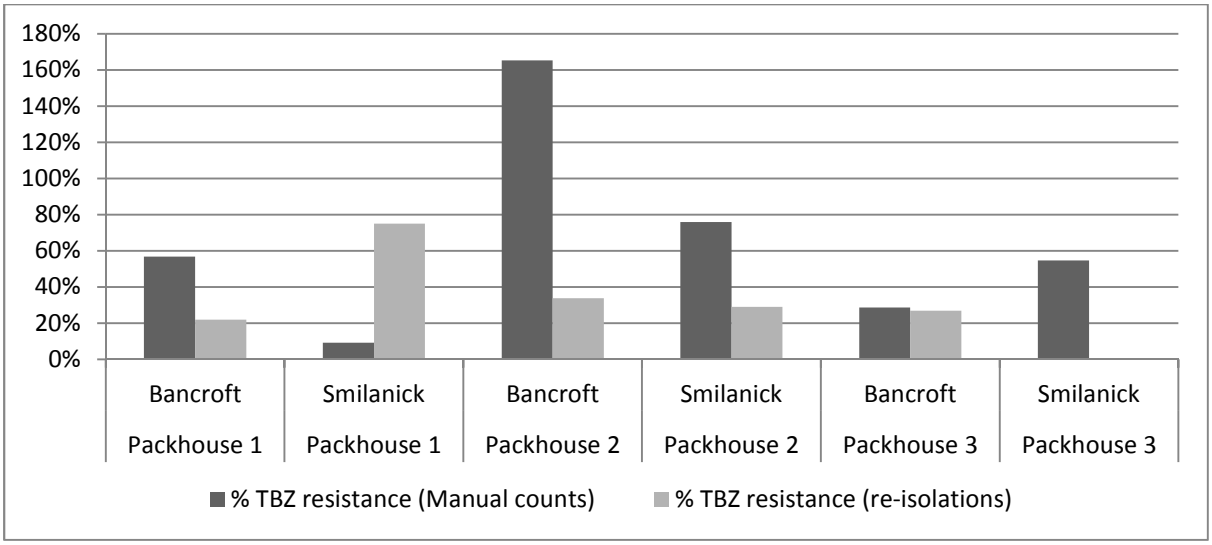

Figure 7. Thiabendazole (TBZ) resistance in *P. digitatum* populations in packhouses as determined by an exposed PDA plate assay, employing different selective media developed by Bancroft *et al.* (1984) and Smilanick and Eckert (1986), and validated by re-isolations onto unamended or 10 µg.mL^{-1} TBZ-amended PDA plates.Exposed plates were incubated for 4 days and validation plates for 7 days at 22°C.

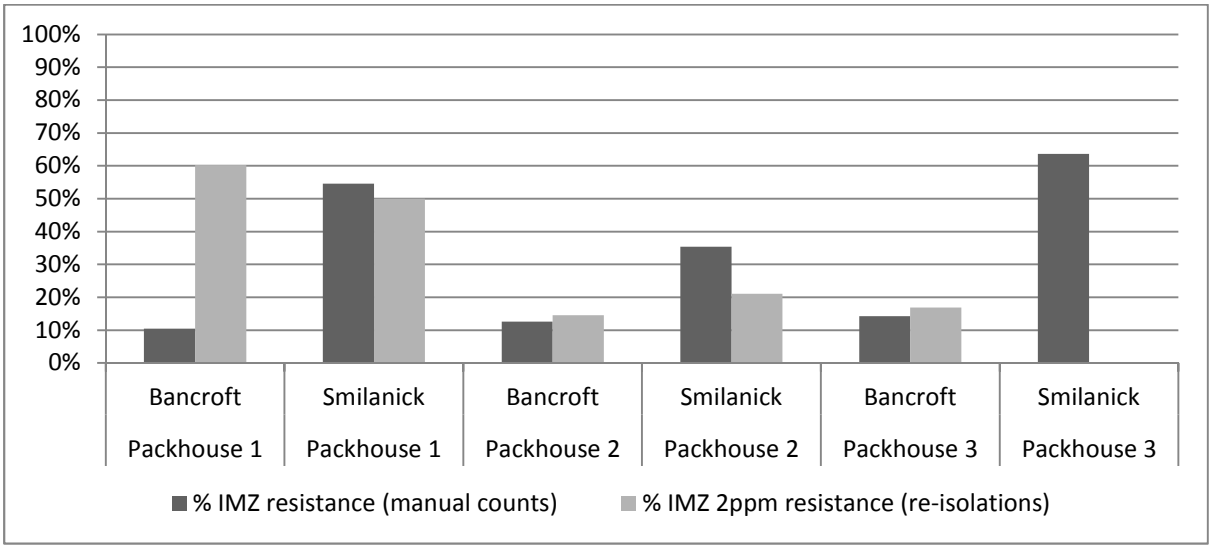

Figure 8. Imazalil (IMZ) resistance in *P. digitatum* populations in packhouses as determined by an exposed PDA plate assay, employing different selective media developed by Bancroft *et al.* (1984) and Smilanick and Eckert (1986), and validated by re-isolations onto unamended or 2 µg.mL^{-1} IMZ-amended PDA plates. Exposed plates were incubated for 4 days and validation plates for 7 days at 22°C.

Annexure A – Analysis of variance tables from Chapter 2

Table 1. Analysis of variance for thiabendazole (TBZ) residues loaded on fruit in different trials where Clementine, navel and Valencia fruit were dipped in TBZ concentrations ranging from 0 – 2000 µg.mL^{-1} for 60 seconds.

Source	Clementine			Navel			Valencia		
	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]
Model	9	0.0806	< 0.0001	9	2.2245	< 0.0001	9	2.855844	<.0001
Trial	1	0.0176	0.0004	1	0.0002	0.9344	1	4.210634	<.0001
TBZ concentration	4	0.1670	< 0.0001	6	3.0358	< 0.0001	6	3.3137	<.0001
Trial* TBZ concentration	4	0.0100	< 0.0001	2	0.0014	0.9456	2	0.804881	0.0137
Error	30	0.0011		18	0.0247		25	0.157193	
Corrected Total	39			27			34		

[a]DF = Degrees of freedom [b]MS = Mean sum of squares [c]P = Probability

Table 2. Analysis of variance of control (%) data of a sensitive or resistant isolate of *Penicillium digitatum* obtained following curative and protective dip treatments of Clementine mandarins, navel and Valencia oranges in different thiabendazole (TBZ) concentrations (0 to 2000 µg.mL^{-1}) and incubation at 22°C for 4-5 days.

Source	Clementine			Navel			Valencia		
	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]
Model	47	35168.8737	< 0.0001	43	73477.0327	< 0.0001	43	259106.1537	< 0.0001
Trial	1	3548.3007	0.0086	1	2735.8156	0.0016	1	206580.6421	< 0.0001
Treatment	5	97384.7298	< 0.0001	6	114073.0387	< 0.0001	6	111104.8782	< 0.0001
Action	1	167168.4578	< 0.0001	1	268429.7316	< 0.0001	1	1095880.5001	< 0.0001
Isolate	1	411369.7871	< 0.0001	1	1453576.0292	< 0.0001	1	40357.6347	< 0.0001
Trial*Treatment	5	510.7485	0.4196	3	451.1744	0.1747	3	64548.7871	< 0.0001
Trial*Action	1	2433.0535	0.0296	1	2508.6700	0.0024	1	6899.6958	0.0008
Trial*Isolate	1	1082.5772	0.1467	1	9785.4298	< 0.0001	1	7766.5266	< 0.0001
Treatment*Action	5	26782.9858	< 0.0001	6	17730.2743	< 0.0001	6	69819.2894	< 0.0001
Treatment*Isolate	5	36760.3443	< 0.0001	6	85545.4981	< 0.0001	6	188185.6697	< 0.0001
Action*Isolate	1	290266.7430	< 0.0001	1	427564.6706	< 0.0001	1	12263.0197	< 0.0001
Trial*Treatment*Action	5	991.2015	0.0862	3	572.9410	0.0980	3	1643.4896	0.0441
Trial*Treatment*Isolate	5	978.6607	0.0902	3	1543.1948	0.0007	3	31597.6192	< 0.0001
Trial*Action*Isolate	1	9479.3154	< 0.0001	1	2307.9258	0.0037	1	19021.4124	< 0.0001
Treatment*Action*Isolate	5	26033.1786	< 0.0001	6	26254.4474	< 0.0001	6	6120.9132	< 0.0001
Trial*Treatment*Action*Isolate	5	2019.4120	0.0015	3	1231.4042	0.0036	3	81071.2810	< 0.0001
Error	2640	513.7076		2452	272.6300		2452	608.2720	
Corrected Total	2687			2495			2495		

[a]DF = Degrees of freedom [b]MS = Mean sum of squares [c]P = Probability

Table 3. Analysis of variance of sporulation incidence (%) data following curative and protective dip treatments of Clementine mandarins, navel and Valencia oranges at different thiabendazole (TBZ) concentrations (0 to 2000 µg.mL^{-1}) inoculated with either a sensitive or resistant isolate of *P. digitatum*. Fruit were incubated at 22°C for 10 - 11 days before sporulation was recorded and incidence (%) calculated.

Source	Clementine			Navel			Valencia		
	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]
Model	47	0.0354	< 0.0001	42	0.6873	< 0.0001	43	0.6734	< 0.0001
Trial	1	0.0222	0.1833	1	0.5621	0.0008	1	0.8191	0.0003
Treatment	5	0.0319	0.0268	6	0.9817	< 0.0001	6	2.0575	< 0.0001
Action	1	0.0035	0.5973	1	2.1816	< 0.0001	1	0.0798	0.2538
Isolate	1	0.0047	0.5395	1	0.2477	0.0263	1	0.7190	0.0006
Trial*Treatment	5	0.0755	< 0.0001	3	0.6463	< 0.0001	3	0.0469	0.5133
Trial*Action	1	0.0103	0.3647	1	0.0856	0.1916	1	0.0143	0.6292
Trial*Isolate	1	0.2216	< 0.0001	1	0.0875	0.1865	1	0.0004	0.9337
Treatment*Action	5	0.0038	0.9110	6	0.5707	< 0.0001	6	0.3188	< 0.0001
Treatment*Isolate	5	0.0769	< 0.0001	6	1.1880	< 0.0001	6	0.6463	< 0.0001
Action*Isolate	1	0.0004	0.8500	1	2.0549	< 0.0001	1	0.0808	0.2509
Trial*Treatment*Action	5	0.0109	0.5016	3	0.4646	< 0.0001	3	0.0170	0.8414
Trial*Treatment*Isolate	5	0.0382	0.0096	3	0.6168	< 0.0001	3	0.0259	0.7365
Trial*Action*Isolate	1	0.0323	0.1087	1	0.0215	0.5123	1	0.2487	0.0441
Treatment*Action*Isolate	5	0.0102	0.5398	6	0.2591	< 0.0001	6	0.2108	0.0022
Trial*Treatment*Action*Isolate	5	0.0074	0.7077	2	0.1307	0.0741	3	0.1102	0.1455
Error	2461	0.0130		1914	0.0501		1881	0.0610	
Corrected Total	2508			1956			1924		

[a]DF = Degrees of freedom [b]MS = Mean sum of squares [c]P = Probability

Table 4. Analysis of variance for thiabendazole (TBZ) residue data following curative and protective carnauba wax amended with TBZ coating treatments on Clementine, Satsuma and Valencia fruit with treatments consisting of coating fruit at three different wax loads (0.6, 1.2 or 1.8 L.ton^{-1}) at a TBZ concentration of 0 or 4000 µg.mL^{-1}, or dipping fruit into an aqueous TBZ suspension of 1000 µg.mL^{-1} (Valencia oranges) or 2000 µg.mL^{-1} (Satsuma and Clementine mandarins) followed by non-amended wax coating treatment of 1.2 L.ton^{-1}.

Source	DF[a]	MS[b]	P[c]
Model	18	12.4952	< 0.0001
Citrus type	2	40.2179	< 0.0001
Treatment	7	17.1503	< 0.0001
Citrus type*Treatment	9	2.7139	0.0009
Error	40	0.6676	
Corrected Total	58		

[a]DF = Degrees of freedom [b]MS = Mean sum of squares [c]P = Probability

Table 5. Analysis of variance for infection (%) and sporulation incidence (%) data on fruit following curative and protective carnauba wax coating treatments of Valencia, Clementine and Satsuma fruit and inoculation with a TBZ-sensitive or TBZ-resistant isolate of *Penicillium digitatum*. Treatments consisted of coating fruit at three different wax loads (0.6, 1.2 or 1.8 L.ton^{-1}) incorporated with a TBZ concentration of 0 or 4000 µg.mL^{-1}. Fruit were incubated at 22°C for 4-5 days before infection was recorded and infection (%) calculated. Fruit were rated for sporulation incidence after 10 – 11 days.

	% Infection			Sporulation incidence		
Source	DF[a]	MS[b]	P[c]	DF[a]	MS[b]	P[c]
Model	71	35910.6	< 0.0001	71	7.207	< 0.0001
Citrus Type	2	196749	< 0.0001	2	5.813	< 0.0001
Treatment	6	50078.9	< 0.0001	6	38.966	< 0.0001
Action	1	7012.22	0.001	1	2.36	< 0.0001
Isolate	1	33195	< 0.0001	1	108.683	< 0.0001
Citrus Type*Treatment (Trt)	12	47529.8	< 0.0001	12	1.199	< 0.0001
Citrus Type*Action	2	21997.4	< 0.0001	2	0.161	0.051
Citrus Type*Isolate	2	159579	< 0.0001	2	0.701	< 0.0001
Trt*Action	6	3459.78	< 0.0001	6	0.359	< 0.0001
Trt*Isolate	6	68944.9	< 0.0001	6	28.171	< 0.0001
Action*Isolate	1	4306.92	0.012	1	0.04	0.388
Citrus Type*Trt*Action	12	2599.79	< 0.0001	12	0.425	< 0.0001
Citrus Type*Trt*Isolate	6	23931	< 0.0001	6	0.693	< 0.0001
Citrus Type*Action*Isolate	2	4643.78	0.001	2	2.937	< 0.0001
Trt*Action*Isolate	6	6114.24	< 0.0001	6	0.508	< 0.0001
Citrus Type*Trt*Action*Isolate	6	4727.28	< 0.0001	6	0.348	< 0.0001
Error	6840	684.102		5969	0.054	
Corrected Total	6911			6040		

[a]DF = Degrees of freedom [b]MS = Mean sum of squares [c]P = Probability

Table 6. Analysis of variance for chilling injury index on Valencia, Satsuma and Clementine fruit that were treated with a wax coating amended with 0 or 4000 µg.mL^{-1} TBZ applied at 0.6, 1.2 or 1.8 L.ton^{-1}, or dipped in a 1000 (Valencia) or 2000 (Satsuma and Clementine) µg.mL^{-1} TBZ suspension and then waxed with non-amended wax at 1.2 L.ton^{-1}. Fruit were incubated at -0.5°C for 40 days and then at 22°C for 4 days before being rated. Two trials were done on each citrus type.

Source	DF[a]	MS[b]	P[c]
Model	29	25336.4352	< 0.0001
Citrus Type	2	200415.1155	< 0.0001
Treatment	9	14902.6042	< 0.0001
Citrus Type*Treatment	18	13112.1126	< 0.0001
Error	2538	1805.8018	
Corrected Total	2567		

[a]DF = degrees of freedom [b]MS = mean squares [c]P = Probability

Table 7. Analysis of variance for green button incidence (%) on Valencia, Satsuma and Clementine fruit that were treated with a wax coating amended with 0 or 4000 µg.mL^{-1} TBZ applied at 0.6, 1.2 or 1.8 L.ton^{-1}, or dipped in a 1000 (Valencia) or 2000 (Satsuma and Clementine) µg.mL^{-1} TBZ suspension and then waxed with non-amended wax at 1.2 L.ton^{-1}. Fruit were incubated at -0.5°C for 40 days and then at 22°C for 4 days before being rated. Two trials were done on each citrus type.

Source	DF[a]	MS[b]	P[c]
Model	29	8.3369	< 0.0001
Citrus Type	2	104.5222	< 0.0001
Treatment	9	1.5927	< 0.0001
Citrus Type*Treatment	18	0.7877	< 0.0001
Error	2538	0.1404	
Corrected Total	2567		

[a]DF = degrees of freedom [b]MS = mean squares [c]P = Probability

Table 8. Analysis of variance for TBZ residue level data on navel oranges following drench treatments, which consisted of a combination of TBZ concentration (1000 or 2000 µg.mL^{-1}) and exposure time (30, 60 or 90s).

Source	DF[a]	MS[b]	P[c]
Model	5	5.0604	0.0439
TBZ concentration	1	21.9864	0.0023
Exposure time	2	1.0851	0.5498
TBZ concentration*Exposure time	2	0.5726	0.7257
Error	18	1.7544	
Corrected Total	23		

[a]DF = Degrees of freedom [b]MS = Mean squares [c]P = Probability

Table 9. Analysis of variance for control (%) data obtained on navel orange fruit following drench treatment with TBZ concentrations of 1000 or 2000 µg.mL^{-1} and exposure times of 30, 60 or 90 s at different orientations (fruit facing up or down). Fruit were treated curatively (inoculated 24 or 6 hours before treatment) or protectively.

Source	DF[a]	MS[b]	P[c]
Model	35	28619.2108	< 0.0001
TBZ concentration	1	5051.0321	0.0158
Exposure time	2	7503.4985	0.0002
Action	2	425564.2163	< 0.0001
Orientation	1	88678.3428	< 0.0001
TBZ concentration*Exposure time	2	312.3804	0.6968
TBZ concentration*Action	2	4907.9107	0.0035
TBZ concentration*Orientation	1	94.8675	0.7405
Exposure time*Action	4	972.6187	0.3430
Exposure time*Orientation	2	634.5709	0.4802
Action*Orientation	2	9146.4927	< 0.0001
TBZ concentration*Exposure time*Action	4	843.7297	0.4195
TBZ concentration*Exposure time*Orientation	2	97.3500	0.8935
TBZ concentration*Action*Orientation	2	109.3344	0.8812
Exposure time*Action*Orientation	4	925.1562	0.3698
TBZ concentration*Exposure time*Action*Orientation	4	82.6523	0.9839
Error	1692	864.5787	
Corrected Total	1727		

[a]Degrees of freedom [b]MS = Mean squares [c]P = Probability

Table 10. Analysis of variance for sporulation incidence on navel fruit following drench treatment, which consisted of a combination of TBZ concentration (1000 or 2000 µg.mL^{-1}) and exposure time (30, 60 or 90 s). Fruit were treated curatively (inoculated 6 or 24 hours before treatment) or protectively. Fruit were placed at different orientations in the drench crate, either facing button-up or button-down.

Source	DF[a]	MS[b]	P[c]
Model	35	0.8292	< 0.0001
TBZ concentration	1	5.4450	< 0.0001
Exposure time	2	5.3686	< 0.0001
Action	2	0.7523	0.0342
Orientation	1	0.4867	0.1392
TBZ concentration*Exposure time	2	0.7367	0.0366
TBZ concentration*Action	2	0.6204	0.0617
TBZ concentration*Orientation	1	0.0469	0.6462
Exposure time*Action	4	0.7402	0.0100
Exposure time*Orientation	2	0.1742	0.4570
Action*Orientation	2	0.4537	0.1303
TBZ concentration*Exposure time*Action	4	0.1620	0.5723
TBZ concentration*Exposure time*Orientation	2	0.6094	0.0648
TBZ concentration*Action*Orientation	2	0.0903	0.6664
Exposure time*Action*Orientation	4	0.0214	0.9837
TBZ concentration*Exposure time*Action*Orientation	4	0.4340	0.0994
Error	1692	0.2224	
Corrected Total	1727		

[a]Degrees of freedom [b]MS = Mean squares [c]P = Probability Probability

Table 11. Analysis of variance for TBZ residue data on Clementine fruit following drench treatments, which consisted of a combination of 1000 µg.mL^{-1} TBZ with exposure times 30, 60 or 90 s, or 2000 TBZ µg.mL^{-1} with an exposure time of 45 s.

Source	DF[a]	MS[b]	P[c]
Model	3	0.4935	0.1933
Treatment	3	0.4935	0.1933
Error	9	0.2540	
Corrected Total	12		

[a]DF = Degrees of freedom [b]MS = Mean squares [c]P = Probability

Table 12. Analysis of variance for control (%) data obtained on Clementine mandarins following drench treatment with TBZ concentrations of 1000 or 2000 µg.mL^{-1} and exposure times of 30, 45, 60 or 90 s. Fruit were treated curatively (inoculated 24 or 6 hours before treatment) or protectively, and placed at different orientations (top or bottom facing upwards) in the crate.

Source	DF[a]	MS[b]	P[c]
Model	23	26653.9742	< 0.0001
Treatment	3	146.8169	0.9075
Action	2	239427.7672	< 0.0001
Orientation	1	45984.7512	< 0.0001
Treatment*Action	6	3026.0947	0.0010
Treatment*Orientation	3	2013.7765	0.0568
Action*Orientation	2	3215.5699	0.0183
Treatment*Action*Orientation	6	812.6891	0.4129
Error	840	799.3563	
Corrected Total	863		

[a]Degrees of freedom [b]MS = Mean squares [c]P = Probability

Table 13. Analysis of variance for sporulation incidence on fruit following drench treatments on Clementine mandarins, which consisted of a combination of TBZ concentration (1000 or 2000 µg.mL^{-1}) and exposure time (30, 45, 60 or 90s). Fruit were treated curatively (inoculated 24 or 6 hours before treatment) or protectively, and placed at different orientations (top or bottom facing upwards) in the crate.

Source	DF[a]	MS[b]	P[c]
Model	23	1.1210	< 0.0001
Treatment	3	3.4367	< 0.0001
Action	2	2.0410	< 0.0001
Orientation	1	0.0002	0.9777
Treatment*Action	6	0.8878	0.0003
Treatment*Orientation	3	0.1455	0.5520
Action*Orientation	2	0.0138	0.9356
Treatment*Action*Orientation	6	0.1847	0.5021
Error	791	0.2077	
Corrected Total	814		

[a]Degrees of freedom [b]MS = Mean squares [c]P = Probability